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Preface

Since its establishment in 1964, the Joint FAO/IAEA Programme of Nuclear Techniques for Food and Agriculture has supported the FAO and IAEA Member States in the use of nuclear technologies in plant breeding and genetics. This has been achieved primarily through R&D and technology transfer of effective methods for mutation induction and mutation detection. Mutation induction in plants aims to generate novel genetic diversity for plant breeders targeting increased yield, improved quality traits, resistance to pests and diseases and tolerance to abiotic stresses such as salinity, drought and heat. Plant mutation breeding has a long track record of success and has impacted agricultural productivity worldwide. To date, more than 3400 mutant cultivars have been registered in the Mutant Variety Database of the Joint FAO/IAEA Division (https://mvd.iaea.org/) as voluntarily submitted records from Member States. These cultivars span more than 220 crop species and represent more than 70 countries. Agriculture in the twenty-first century faces multiple challenges, including food security, the urgent need for more sustainable agricultural production methods and adaptation to climate change. Plant mutation breeding is well positioned to help address these global challenges due to the broadening of germplasm selection pools by inducing novel genetic variation.

Induced genetic variation and mutation breeding have been recently applied to the development of resistance in cereal crops to the parasitic weed, *Striga. Striga* is the genus for several species of parasitic weeds that plague cereal crops including maize (*Zea mays*), upland rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*). The major *Striga* species affecting these crops are *S. asiatica* and *S. hermonthica.* Other species, including *S. gesnerioides*, parasitize legumes, especially cowpea (*Vigna unguiculata*). In this book, we use *Striga* to refer mainly to *S. asiatica* and *S. hermonthica.* A Coordinated Research Project (CRP) titled *Mutation Breeding for Resistance to Striga Parasitic Weeds in Cereals for Food Security* (D25005) was undertaken by the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture from 2016 until 2021 to investigate if physical mutagenesis can generate useful genetic variation for resistance to *Striga* in the major cereal crops, maize, rice and sorghum (http://cra.iaea.org/cra/stories/2016-01-18-D25005-Resistance-to-Striga.html).

This book describes the results of this CRP and provides an overview of the use of physical mutagens to induce *Striga* resistance in cereal crop plants cultivated in sub-Saharan Africa where the parasitic weed poses a major biological constraint to grain yield. Examples of mutation breeding to improve *Striga* resistance through gamma ray induction are presented for maize, rice and sorghum in three IAEA Member States, Burkina Faso, Madagascar and Sudan. Each chapter authored by researchers from these Member States describes the details of screening for resistance to the parasitic weed and derivation of mutant lineages for improved *Striga* resistance relative to their respective unmutagenized starter lines. These individual cases are followed by protocols aimed at characterizing the mechanisms of resistance and ultimately the causal genetic changes. Efficiency enhancing technologies including genomic analysis, rapid cycling of plant generations and doubled haploids were integrated in the mutation breeding process to accelerate the delivery of *Striga* resistant cereal mutants. We hope that the book will be useful to the international community in their endeavour to limit the adverse effects of *Striga* on food security.

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Contents

Introduction to Mutation Breeding in Cereal Crops for Resistance to *Striga*

Striga as a Constraint to Cereal Production in Sub-Saharan Africa and the Role of Host Plant Resistance

Patrick J. Rich

Abstract This chapter provides an overview on the biology of the parasitic weeds of the genus *Striga*, specifically *S. asiatica* and *S. hermonthica*, and their impact on three cereal staples of sub-Saharan Africa, maize, rice and sorghum. Host plant resistance to *Striga* mitigates the yield losses to these crops. Improvement of *Striga* resistance in these three crops is discussed including the possibility of expanding resistance sources through mutagenesis.

Keywords *Striga* · Maize · Rice · Sorghum · Resistance

Striga and Its Parasitic Relationship with Cereals

Through their evolutionary course, some plants have lost their autotrophic nature and depend on other plants to provide them with the water and nutrients they require to complete their life cycle. These parasitic plants account for approximately 1% of angiosperms, totalling over 4000 species distributed in over 30 families (Nickrent 2018). Most parasitic plants are curious members of natural ecologies rather than agricultural pests. A few, like sandalwood, are even valuable crops themselves. Some, however, have adapted to crop hosts and have therefore become weeds. *Striga* is a genus name in the family Orobancaceae which contains 1725 parasitic plant species (Nickrent 2018). *Striga* itself includes approximately 40 species, all hemiparasitic, meaning that they have retained some photosynthetic capacity, but all obligate parasites, requiring a host plant to survive through maturity. Most *Striga* species parasitize grass hosts with *S. gesnerioides*, a parasitic weed in cowpea, a notable exception. The name *Striga* is the Latin word for witch. *Striga* are therefore commonly known as witchweeds in English with similar common names in the languages of many sub-Saharan African peoples that inhabit their native range. The name is descriptive of the

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syndrome cereals display under *Striga* infestation. Even before the weed emerges and transforms a grain field into a sea of red or purple flowers, the crop appears to be under a spell that robs it of its verdancy. The most notorious *Striga* species are *S. asiatica* and *S. hermonthica* and it is to these which we refer throughout this book as *Striga. Striga* causes an estimated seven billion USD in crop productivity losses annually across sub-Saharan Africa (Yacoubou et al. 2021). *Striga* are in many agroecologies the primary biological constraints to maize, rice and sorghum production. There are few control options among these crops. These include host plant genetic components that mitigate the degree of *Striga* infestation (resistance) and impact of infestation on crop productivity (tolerance). Host plant resistance and tolerance are considered vital components of *Striga* management strategies, but unfortunately sources are rare and largely uncharacterized among these staple grain crops (Rich 2020). Yield losses to *Striga* are concentrated among the subsistence farms where agricultural inputs that can reduce their impact (e.g., irrigation, fertilizer and even improved cultivars) are rarely used. It is with view to these farmers that this mutagenesis project was undertaken.

In order for the parasite to succeed on its host, it must cue its development to a series of vulnerabilities manifested by the cereal during its growing season. These are described in more detail in Chapters "Physical Mutagenesis in Cereal Crops"–"An Agar-Based Method for Determining Mechanisms of Striga Resistance in Sorghum" and elsewhere (see Rich 2020), but briefly involve germinating in response to chemical stimulants (strigolactones) exuded by the host seedling root. Next, the *Striga* radicle tip must form a haustorium (organ of attachment and acquisition) at or very near an actively growing host in response to 2,6-dimethoxybenzoquinone (DMBQ) and other by-products of secondary cell wall formation. The haustorium must then attach to the host root and penetrate the epidermis, cortex, endodermis and xylem elements, all the while evading any host defences that could halt its progression. Further haustorial development must then occur until a functional vascular union is achieved with its host, ensuring a sustained flow of water and nutrients to support *Striga* shoot development and sufficient growth to the point of emergence, flowering and seed production. A healthy *S. hermonthica* plant can produce in excess of 100,000 seeds and these seeds can remain viable for a decade or longer.

Resistance traits can be anything that interferes with establishment of this parasitic relationship, rendering a host less compatible to *Striga* infection at any or multiple points of this process such that the number or vigor of successful parasites is reduced relative to that on a susceptible, or more compatible host. Reducing the number of successful parasites protects both the current and future crops. Beyond the effects of resource theft, *Striga* is notorious for further negative effects on host plant fitness, things like increased root growth at the expense of shoot growth, chlorosis and leaf senescence, which in crop plants translates to severe or even total reductions in grain yield. Tolerance traits affect a host's ability to be less sensitive to these peculiar toxic effects of *Striga* infestation and thereby sustain grain production in comparable amounts to uninfested plots.

Striga on Maize

Although maize (*Zea mays*) is an introduction from the Americas, it is today the major cereal crop in both area under cultivation and grain production in Africa (Rich and Ejeta 2008). *Striga* is a major biological constraint to maize cultivation across most areas of sub-Saharan Africa (SSA) and thereby reduces food security of millions of farmers and those that depend on the grain they produce (Yacoubou et al. 2021). There are limited control options and most of these are not readily available to subsistence farmers, by far the most numerous in *Striga* endemic areas. Integrated approaches employing both resistant varieties along with *Striga* management practices are generally more effective than singular control methods, but again, too costly or unavailable to most maize farmers in SSA (Rich 2020; Yacoubou et al. 2021). Sources of genetic resistance to *Striga* are rare and come largely from maize's wild relatives, *Tripsicum dactyloides* and *Zea diploperennis* (Gurney et al. 2003; Amusan et al. 2008). Other sources include landraces, inbred lines, open pollinated varieties and some hybrids (Yacoubou et al. 2021). Tolerance, (reduced impact of *Striga* infestation on yield) is used more in maize than genetic resistance (host plant factors that reduce *Striga* infestation) because the former is more widely available (Yacoubou et al. 2021). However, tolerance does little to control the spread of *Striga* among maize production areas and pest populations can quickly overwhelm these lands to the point where they are no longer suitable for maize cultivation (Yacoubou et al. 2021). Known resistance mechanisms in maize include low *Striga* germination stimulant activity of root exudates (Gurney et al. 2003; Adetimirin et al. 2000; Karaya et al. 2012), low haustorial induction (Gurney et al. 2003; Mutinda et al. 2018), reduced attachment due to reduced host root branching (Amusan et al. 2008), reduced number of successful penetrations (Gurney et al. 2003; Amusan et al. 2011) and escape through early maturity (Oswald and Ransom 2004). The genetic basis underlying these resistance mechanisms remains poorly understood. No known genes specifically controlling resistance/susceptibility have been identified and therefore markers for *Striga* incompatible alleles do not yet exist (Yacoubou et al. 2021).

Striga on Rice

Although one species of rice (*Oryza glaberrima*) was domesticated in West Africa nearly 3000 years ago, the predominant species cultivated across sub-Saharan Africa is now Asian rice (*O. sativa*) introduced through East Africa from India 500 years ago and is today the second most important cereal on the Continent (Zenna et al. 2017). A group of rice varieties called NERICA (New Rice for Africa) developed at the Africa Rice Centre in East Africa from crosses between the native and introduced rice species are promising sources of *Striga* resistance (Rodenburg et al. 2017). Genomic regions in rice associated with *Striga* resistance have been reported. One of the progenitors of the mapping population used to identify quantitative trait loci (QTL) for *Striga*

resistance was a line that showed a strong incompatible reaction to *S. hermonthica.* Parasites were unable to establish vascular connections with the rice host (Gurney et al. 2006). From testing the mapping population under *Striga* infestation, four QTL with major effects on resistance to *S. hermonthica* were identified. Expression profiling was used to find three candidate genes coding for uncharacterized proteins within one of the major QTL associated with resistance (Swarbrick et al. 2008). Quantitative trait loci with major effects on tolerance to *S. hermonthica* have also been reported in rice (Kaewchumnong and Price 2008). Mechanisms of low *Striga* germination stimulant activity and incompatibility were characterized among rice cultivars with field resistance to both *S. asiatica* and *S. hermonthica* (Samejima et al. 2016; Rodenburg et al. 2017). In one of these, a gene involving regulation of salicylic acid and jasmonic acid defense signaling pathways was found to condition the resistance (Mutuku et al. 2015).

Striga on Sorghum

Sorghum was domesticated in the same parts of Africa where *Striga* is believed to have originated (Rich and Ejeta 2008). It is perhaps because the two species share a common geological origin that *Striga* resistance in sorghum is better defined than in maize and rice.

In sorghum, low *Striga* germination stimulant activity is a useful source of resistance. Inheritance of this trait is through recessive alleles (*lgs1*) at a locus named *LOW GERMINATION STIMULANT1* (Gobena et al. 2017). Sorghum varieties homozygous for *lgs1* generally support fewer *Striga* plants relative to susceptible varieties. The *LGS1* gene codes for a sulfotransferase unique to sorghum that controls the stereochemistry of the strigolactones during the final step of their biosynthesis (Yoda et al. 2021). That stereochemistry determines the *Striga* germination stimulant activity of the strigolactones that dominate the sorghum root exudate (Gobena et al. 2017). *LOW GERMINATION STIMULANT1* is currently the only known *Striga* resistance gene in any cereal.

Exudation of germination inhibitors is a possible *Striga* resistance trait in sorghum, although the chemical identity of these inhibitors remains largely unknown (Weerasuriya et al. 1993; Rich et al. 2004). Reduced haustorial inducing capacity of sorghum root exudates is another possible *Striga* resistance trait in sorghum. Complete lack of haustorial initiation factors released by growing roots is unlikely, given that DMBQ is a by-product of host root growth through elongation. Certain sorghum lignin mutations called *brown midrib12* (*bmr12*) specifically reduce syringyl components of secondary cell wall lignin (Saluja et al., 2021). These syringyl lignin subunits are direct precursors to DMBQ (Cui et al. 2018). A preliminary test of haustorial inducing capacity of sorghum *bmr12* mutants with *S. hermonthica* showed 20% fewer haustoria formed near the roots of these mutants in agar than occurred in the presence of wildtype sorghum roots (Rich 2018). Whether *bmr12* sorghum is less parasitized

in a soil environment remains to be tested. We have also observed lower haustorial initiation capacity among certain wild sorghum accessions (Rich et al. 2004).

Root characteristics like decreased branches may result in fewer *Striga* attachments through avoidance of potential parasites. We have observed in our various co-culture laboratory methods that *Striga* are more likely to attach to and successfully penetrate thinner root branches than on the primary roots of sorghum seedlings. The altered lignin mutation *bmr12* also causes root architectural changes resulting in fewer branches in the upper soil profile with respect to wildtype (Saluja et al. 2021). Several other QTLs have been identified in sorghum that control root architecture (Parra-Londono et al. 2018) and certain alleles that specifically condition fewer root branches in the upper soil profiles may therefore contribute to *Striga* resistance through avoidance.

Sorghum has a reputation for producing allelopathic chemicals like sorgoleone produced in its root hairs (Głąb et al. 2017). Sorgoleone is a potent phytotoxin, inhibiting multiple vital processes impacting photosynthetic, root and mitochondrial functions (Dayan 2006). Phytotoxicity toward *Striga* has not been specifically studied. If sorgoleone or the other allelopathic compounds present in sorghum root exudates have an antibiotic effect on *Striga*, they would likely protect it at the pre-attachment phases of the life cycle. These and other components of sorghum root exudates might also act indirectly by influencing the microflora of the rhizosphere favoring *Striga*-suppressive rhizobacterial or mychorrhizal species (Schlemper et al. 2017).

A number of post-attachment resistance reactions have been described in sorghum that stops the parasite before vascular connections are established. One of these is an apparent hypersensitive response that shows reddening and necrosis in host root epidermal and cortical cells surrounding the attachment site, generally isolating the invading tissues and blocking parasite establishment. The response was described in derivatives from wild sorghum (*S. bicolor* × *S. b. verticilliflorum*) challenged with *S. asiatica* in laboratory co-culture and is inherited through dominant alleles at two loci named *Hrs1* and *Hrs2* (Mohamed et al. 2010). This defence response appears similar to the hypersensitive response characterized in cowpea against *S. gesnerioides* and may be triggered by as yet unidentified effectors from the parasite (Li and Timko 2009). Other reactions generally described as "mechanical barriers" have been reported in resistant sorghums upon attachment of *S. asiatica* expressed in the cortex and endodermis that prevent invading parasite tissue from reaching the vasculature (Maiti et al. 1984). We have used the term incompatibility to indicate the collective post-attachment resistance responses that do not have obvious host-tissue necrosis (Pérez-Vich et al. 2013). These may include several mechanisms controlled by multiple loci. The overall effect is to arrest or reduce the rate of successful parasitic events. In sorghum infected with *Striga*, these are usually not 100% protective, that is, some parasites on resistant varieties usually do manage to emerge and set seed, but the frequency of these events is reduced relative to the numbers of successful parasites on susceptible varieties. Incompatibility may be expressed in the cortex, at the endodermis or even after penetration of xylem vessels. Attached *Striga* in these instances are slow to develop and often die before reaching maturity. Incompatible reactions

are expressed in host tissues as extra thickening of the endodermis and deposition of phenolic compounds at the interface with haustorial cells or even occlusion of vessels where the parasite initially breached its xylem elements (Maiti et al. 1984; Arnaud et al. 1999; Amusan et al. 2011; Mbuvi et al. 2017). The haustoria of *Striga* infecting incompatible sorghum often appears diminished relative to those of successful parasitic events. These symptoms may represent instances of active defence responses triggered by the parasite or simply a constitutively unsupportive cellular environment, perhaps lacking key metabolites preferred by the parasite to establish and grow. Unfortunately, none of these post-attachment reactions in sorghum to *Striga* have been so precisely characterized and exploited in resistance breeding as *lgs1*, and markers specific to only *lgs1* currently exist.

Potential Contribution of Mutagenesis to Improved *Striga* Resistance

Multiple resistance traits, even those that render a plant slightly incompatible with *Striga* establishment, combined in a crop genotype are more sustainable than individual resistant traits since a parasite population would need to accumulate multiple virulence mutations to overcome them. Tolerance too is ideally used in combination with resistance for long-term durable protection. The challenge of deploying these host characters in a sustainable combination is that they are rare, almost never singularly effective (there are no credible reports of *Striga* immune varieties in these crops) and their genetic basis is poorly understood. Adding to these fundamental limitations for the use of host resistance/tolerance, access to *Striga* resistant varieties and inputs that extend their effectiveness by the neediest farmers is extremely limited.

It is in the context of these great challenges that this CRP offers a contribution. We wanted to create through mutagenesis more genetic variants that offer some degree of *Striga* resistance. With view specifically to farmer acceptance of any new resistant varieties that might result from this endeavour, each participating Member State chose a crop variety popular among subsistence farmers with several desirable qualities, but lacking *Striga* resistance, in which to conduct the mutation breeding. Since very few genes are known among the target crops that control *Striga* resistance, a process that caused genome wide changes seemed worth trying. The goal was to identify among the mutagenized lineages *Striga* resistant progeny in farmer-preferred varieties. Useful gained resistance should come as an attribute without yield costs or loss of other desirable characteristics. The resulting germplasm would then have added value as a cultivar itself, at least as a demonstration that a favourite genotype can be genetically protected against the ills of *Striga*. More appropriately in terms of sustainability, the germplasm would serve as starting material for further resistance improvements based on the introgression of other known *Striga* resistance alleles from crosses with donor sources or through gene editing. The fruit of this gained resistance would be even greater if the underlying mutations that caused the improved

Striga resistance can be identified through genomic analysis and thereby become new targets for natural allele mining or gene editing.

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Physical Mutagenesis in Cereal Crops

Abdelbagi M. A. Ghanim

Abstract The use of physical mutagens to induce heritable genetic variation in crop plants dates back to the beginning of the twentieth century. While X-rays were the first to be used for mutation induction in plants, gamma-rays have been the most widely used physical mutagen. Currently gamma induced mutations represents 60% of the registered mutant varieties in the Mutant Variety Database of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture. Beside gamma and X-rays, other physical mutagens include neutrons, beta particles, alpha particles, protons and ion beam. This chapter introduces the technique of physical mutagenesis with emphasis on gamma-ray and X-ray irradiation of seeds in cereals in the context of inducing genetic variation for resistance to the parasitic weed, *Striga*. Easy to follow step-by-step protocols are explained including sample preparation, treatment application and post-treatment handling of irradiated seeds. Data collection and graphic illustration are presented to estimate the optimum dose for bulk treatment to determine the radio-sensitivity of cereal crops. The last section briefly explains the development and handling of mutant populations by way of introduction to the rest of this book on mutation breeding in cereals for resistance to *Striga*.

Keywords Mutation induction · Mutagenesis · Radio-sensitivity · Cereals

Introduction

Mutations are sudden heritable changes in the genome, either spontaneous or induced, that enhance genetic diversity allowing evolution and adaptation of organisms to changing environments. The main objective of mutation breeding is to improve well-adapted farmer-preferred varieties through correcting a specific limitation related to

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productivity, resistance to biotic or abiotic stresses, or aspects of crop quality. Spontaneously occurring mutations are the main drivers of evolution in crop plants. Selection for natural variants with desirable phenotypic impacts on crop quality is the essence of plant breeding. The sum of these mutations accumulated through eons of evolution in the gene pool constitute the genetic diversity of crop species and their wild relatives. The broader this diversity, the greater potential gains in crop adaptability and human guided improvements. Induced mutation has expanded the genetic diversity of crop species and contributed to the development of new and improved crop varieties. Much of the early work in plant mutagenesis was described using X-ray irradiation in maize and barley, and was pioneered by Stadler (1928a, b, 1930). Physical mutagens include gamma and X-rays, neutrons, beta particles, alpha particles, protons and ion beam. Gamma-ray irradiators are the most widely used for the irradiation of plant materials for varietal improvement. The Plant Mutant Variety Database (http://mvd.iaea.org/) of the Joint FAO/IAEA Centre of Nuclear Applications in Food and Agriculture currently holds the records of over 3300 released mutant varieties in a wide range of crop plants of which more than 80% involve the use of ionizing radiation, especially gamma-rays. Tissue penetration depends on the nature of the radiation and this has consequence on the induction of mutations in the DNA. Ionizing electromagnetic radiation such as gamma and X-rays have relatively less biological effectiveness than ionizing atomic particle radiation such as neutrons but have more biological effectiveness than irradiation sources such as UV-light. Relative to X-rays, gamma-rays with their shorter wavelength penetrate tissue deeper. Hard X-rays have deeper tissue penetration than soft X-rays due to their shorter wave length and are hence preferred for use in mutation induction. Changes in DNA caused by gamma-rays include single or multiple nucleotide changes, small insertions and deletions (Yang et al. 2019), larger structural variations due to double-strand breaks and activation of transposable elements (Nielen et al. 2018). Generally, to induce mutations in plants, the planting materials such as seeds and vegetative propagules are exposed to the mutagenic agent. The choice for the appropriate dosage of radiation to be applied depends on the specific plant material, its genetics, and its physiology. By way of introduction, this chapter describes the process of physical mutagenesis with emphasis on gamma and X-ray irradiation, the most widely used mutagens, especially in developing countries. We describe in this introductory chapter all aspects of mutagenesis including the preparation of the seed material, dose optimization, irradiation treatment, post-treatment and handling of the mutant population.

Protocols

Dose optimization. Prior to bulk irradiation, the optimal dose must be determined. The optimum dose of a mutagen is the dose that achieves the optimum mutation frequency with minimal unintended effects. This is usually taken arbitrarily as the dose that causes 50% reduction of vegetative growth of the treated material as compared to the control (GRD_{50}), or the dose that kills 50% of the treated seeds (LD_{50}). In the case

of physical mutagenic sources, the optimal dose is determined through the conduct of a radio-sensitivity test (RST), which is a relative measure indicative of the extent of recognizable effects of radiation exposure on the irradiated material. It helps to determine the optimum dose of irradiation required for a specific plant material for a specific outcome.

Dose uniformity is highly critical for the mutation induction process, both for conducting the RSTs to estimate the optimum dose and for effective bulk treatment. For RSTs, small samples are treated with a wide range of doses and the effect is measured in the form of a reduction in germination or growth as compared to a control (untreated sample). The parameters measured in the RSTs are used to estimate the lethal doses for 50 and 30% reduction of the germination (LD_{50}, LD_{30}) and growth (GR_{50}, GR_{30}) relative to the control. These estimated values enable the determination of the optimum dose(s) for the bulk mutagenesis treatment. It is very important in RSTs to distinguish effects resulting from the irradiation from differences due to variations in the quality of the seed source such as germinability determined from the control.

Dose uniformity test and safety considerations. Dose uniformity in width and depth of the sample is also taken into consideration for dose optimization. The desired irradiation dose may differ from the actual dose received by the irradiated samples, the latter being defined as the absorbed dose. It is important to accurately determine the absorbed dose after irradiation, especially in X-ray irradiation where the power supply might cause fluctuation in the production of the X-rays. A GAFchromic® dosimetry system offers a convenient means to accurately quantify the absorbed dose through the use of a radio-chromic film which changes colour depending on the irradiation doses. One or more films placed at different positions near the sample(s) will allow assessment of the absorbed dose and also the dose uniformity. The GAFchromic® films can be read by a film reader to determine the actual dose applied at the sample level and the uniformity of the treatment across the sample material after each exposure (Fig. 1).

Physical irradiation carries the potential risk of exposure of the operator to ionizing radiation which is mutagenic and carcinogenic. The operation should be handled by trained, authorized personnel in a specialized facility. There is no expected harm from handling of the irradiated seeds and the samples can be handled immediately

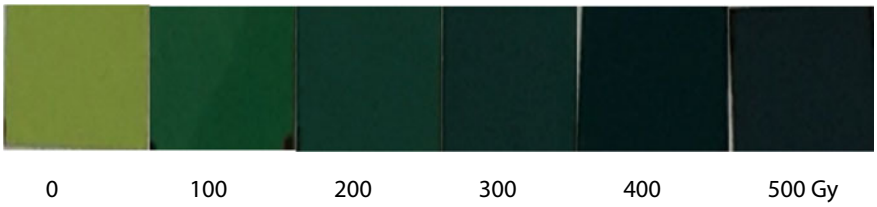

Fig. 1 Films used for the GAFchromic® dosimetry measurement after physical irradiation showing changes in colour from light green to dark with the dose rates, from 0, 100, 200, 300, 400, 500 Gy

after the treatment is completed. The treated seeds may be planted immediately or stored in appropriate conditions until the planting time.

Pre-treatment preparation of seed samples. A source of pure and viable seed is critical for a successful mutation breeding programme, once the target variety/genotype has been selected. This may sometimes require seed multiplication. Fresh breeders or foundation seed is preferably used for mutagenesis. Seeds should be multiplied in sufficient isolation to prevent out-crossing and mixing with other seed sources of the same species. Harvested fresh seeds should be separated from chaff, broken and shriveled seeds and disinfected to eliminate microbial contamination if needed.

Seed viability test. Seed viability is tested before proceeding to the irradiation treatment. This can be done by placing a small sample (10–20 seeds) on a moist filter paper in a petri-dish or other appropriate germination test media. For each specific plant species under experimentation, it is important to follow the recommended conditions for seed germination including breaking of dormancy as needed, seed moisture content, imbibition and incubation temperature/light and duration. Germination is scored after 5–7 days (for small grain cereals), or as appropriate, on the germination media. An example for germinating sorghum seeds is given in Chapter "An Agar-Based Method for Determining Mechanisms of Striga Resistance in Sorghum" and for rice in Chapter "Histological Analysis of Striga Infected Plants". It is recommended to proceed to irradiation treatment only with seeds having a germination percent above 90% (Fig. 2).

Preparation of seed samples. Once seed viability is determined to be above 90%, the seed can be prepared for RSTs. Determine the number of seeds available for use in RST while ensuring that sufficient amount of seed is available for the bulk irradiation. Ideally 15–20 seeds are packed separately in paper envelopes for each dose treatment for RST (Fig. 3). The number of dose treatments ranges between six and ten depending on the space available for planting and on the range of the dose treatments to be applied. Note that the amount of seeds per treatment and the number of treatments will vary with the crop and the growing media (field, pots, trays, petri-dishes, etc.). At the Plant Breeding and Genetics Laboratory (PBGL), we routinely apply six to seven treatments including an untreated (non-irradiated)

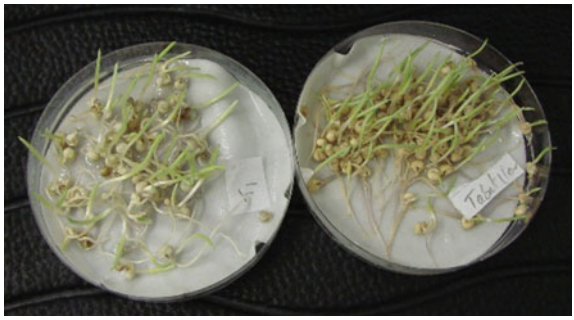

Fig. 2 Representative seed viability test of two sorghum cultivars. Germination percentage after 7 days was > 95%

Fig. 3 Photos from left to right, seed counting and packing in paper bags, labelling the bags and assembling bags of the same treatment in one bigger bag labelled with the dose treatment; placing the bags in a desiccator with 60% glycerol for 3–7 days for moisture equilibration to 12–14%

sample (control). Treatments are usually replicated two to three times depending on the availability of seeds and planting space.

Seeds are labelled for each treatment with the name of the cultivar/genotype, replication number, dose amount, source of irradiation and date of packing (Fig. 3). Seed bags of the same dose treatment are grouped and packed together with the seeds well distributed at the bottom and labelled with the dose and replication. An untreated bag (control) with the same number of seeds is held under the same conditions as the treated ones.

The packed seeds are then placed in a vacuum desiccator with 60% glycerol for moisture equilibration (Fig. 3). The seeds are retained in the desiccator for three to seven days. This equilibrates the seed moisture content of cereals to approximately 12–14%, which is the ideal moisture content for efficient induction of mutation. Water mitigates irradiation through both absorbing radiation and forming free radicals which are directly involved in certain DNA modifications (Spencer-Lopes et al. 2018). Note that critical seed moisture content for mutation induction may vary among plant species. Seed moisture content may be adjusted based on a preliminary test for moisture equilibration in different glycerol concentrations and incubation periods. In addition to the gravimetric method for seed moisture determination using standard oven drying, different types of moisture analyzers are commercially available with varying degrees of accuracy, and both destructive and non-destructive protocols.

Seed irradiation treatment. After moisture equilibration, seeds are removed from the desiccator and transferred to the irradiation facility. Details and description of various irradiation sources and machines used at the PBGL are well described in the third edition of the *Manual on Mutation Breeding* (Spencer-Lopes et al. 2018). Seed treatment for the widely used gamma-ray and X-ray machines specifically designed for mutagenesis are briefly presented here.

Gamma irradiation. The facility is strictly handled by designated technicians. The operator calculates the time needed for exposure by the assigned treatments based on the current dose rate of the source cell. At PBGL, the ^{60}Cobalt cell has a current dose range of 130 Gy per minute. This changes over time as the half-life of ^{60}Co is 5.3 years. Gamma-rays are continually emitted by decay of the cobalt isotope to

nickel but these are contained within the holding chamber by the radiation absorbing material composing its walls. The operator switches on the machine that lowers the sample compartment into the source chamber, sets the exposure time and places the samples one at a time in the sample compartment. Exposure is relatively rapid and involves remote mechanical lowering of the sample compartment into the ^{60}Co source chamber. The irradiation time is calculated by the following equation: Time [min] = (Dose [Gy])/(Dose rate [Gy/min]). Exposure times are typically less than a few minutes. Once the treatment is completed the operator delivers the samples to the requestor.

X-ray irradiation. Like gamma-rays, X-rays are non-particulate electromagnetic radiation emitted as photons but with longer wavelengths than gamma-rays, in the range of 0.01–1 nm. They most commonly cause mutation through breaking DNA strands which are then faultily repaired by cells resulting in genomic deletions (Spencer-Lopes et al. 2018). Because they are much less energetic than gamma-rays, X-rays deliver a much lower dose of radiation, on the order of 2–10 Gy/min. X-ray sources are from special tubes designed to emit radiation upon electrical input. X-ray tubes therefore offer an advantage over gamma-ray generators that rely on certain decaying isotopes in that they can be turned on and off at will through an electrical current. Exposure to reach optimum dose, however, takes much more time than that for gamma-rays. X-ray tubes are also prone to overheating and therefore rely on cooling systems during the long exposure times (up to one hour) required for mutagenic seed irradiation.

The machinery housing the X-ray tube varies, with most designed for medical radiography. X-ray machines therefore most commonly use a movable X-ray tube around a stationary target. Some of these can be adapted to irradiate seeds but require extensive testing to position seeds properly and adjust exposure times to achieve optimal dosage. Other machines, like the RS2400® (RAD Source Technologies Inc., USA) held at the PBGL, are specially designed for mutagenesis and place the target in rotating canisters orbiting a stationary X-ray tube. Seed packets are placed in these canisters, packed in inert parboiled rice and exposed in a preset program at a particular voltage and current for a predetermined time (up to one hour) to achieve the optimum dose. Aluminum shields (0.5 nm) are often used to absorb "soft" X-rays (0.1–1 nm) emitted by the X-ray tube so that only the more mutagenic "hard" X-rays (0.01–0.1 nm) reach the seeds. As with gamma irradiation, seeds are safe to handle after removal from the X-ray machine. The irradiated samples (now called M_1 seeds), together with the untreated control, are transferred to the field, greenhouse or laboratory for planting.

Post-treatment handling of irradiated seeds. The irradiated seeds should be sown as soon as possible after irradiation to minimize post-irradiation damage. If needed, the treated seeds can be stored for a short period of time. For example, in the case of cereals, the seeds may be stored at room temperature for a maximum of four weeks. In the case of longer storage, vacuum packing is used, and storage should be done in the dark and under low-temperature conditions (2–5 °C). These conditions minimize metabolic activity and cellular degradation. Prolonged storage of seed materials after radiation treatment may confound mutagenic effects with ordinary aging.

For planting, the irradiated seeds are transferred to the site of sowing which can be a greenhouse in trays, pots or in any suitable containers, or a laboratory in petri dishes or sandwich blotters, or directly in a field. The choice among these planting media depends on available resources, type and number of treated seeds and mutagenesis goals. In the case of greenhouse planting, the appropriate soil mixture is prepared to ensure optimum drainage, maintain sufficient moisture to the germinating seeds and avoid water logging, e.g., by drilling small holes in the bottom of the plastic trays.

Soil preparation. At the PBGL, local top soil is mixed with commercial sand and peat (Fig. 4) in a 60:25:20 kg ratio. The soil mixture is then distributed and levelled in the plastic sowing trays. An 11.5 × 36.5 × 56.5 cm tray is ideal for a RST involving six doses and 20 seeds per dose in the case of small-grain cereals or similar sized seed.

Assessment of germination and growth rate. Seeds are sown on a well-levelled soil surface after watering the soil slightly to make it uniformly moist. Appropriate multiple-line dibbers are used to ensure uniformity in sowing depth and spacing in order to distribute seeds evenly and to separate samples by dose treatment (usually six furrows). Treatment labels are distributed along the furrows, starting with the control, then incrementally from the lowest to the highest dose treatment, which facilitates visual observation. Envelopes containing the three or two replicates of irradiated seeds are distributed, starting with replicate one, preferably in separate trays. They can be combined if using large trays (Fig. 5). The seeds are covered with a thin layer of dry soil and gently showered with enough water to ensure uniform germination. It is important that watering is optimal. Avoid over-watering or drying to ensure healthy seedling establishment and growth. The plant species and the growing conditions will determine the frequency of irrigation.

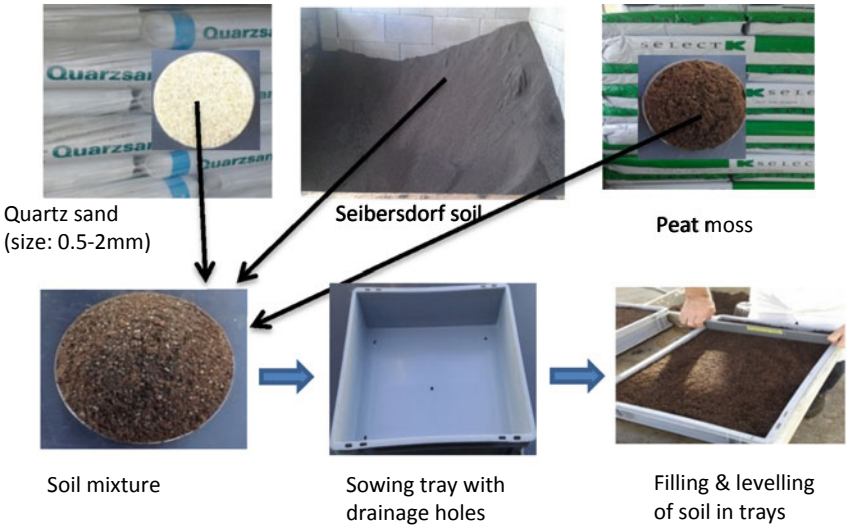

Fig. 4 Mixing soil components and making a seed bed for radio-sensitivity testing

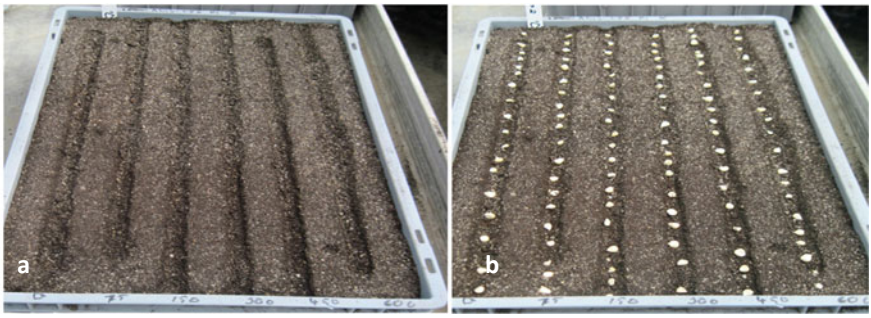

Fig. 5 Plastic trays for assessing radiation effects on maize seeds. **a** Planting furrows for irradiated maize seeds in the order of increasing dose from left to right (0 = control, 75, 150, 300, 450 and 600 Gy). **b** Even sowing of M_1 seeds before covering with dry soil and then watering

Germination data is collected starting at about 5–7 days after sowing in the case of cereals. Plant height is recorded after two weeks or otherwise, as appropriate, depending on the plant species. It is critical to avoid delays in recording growth-related data as some plant species may recover with time from the damage caused by irradiation.

Data collected on germination or growth rate, e.g., plant height or root length, is used for estimation of the optimum dose. This optimum dose is based on calculations on seedling survival that produce 50 or 30% (LD_{50}, LD_{30}) lethality, or seedling growth reduction based on shoot and/or root growth reduced by 50 or 30% (GR_{50}, GR_{30}) relative to the control (Fig. 6). The radio-sensitivity data based on both seed germination and seedling characteristics/growth are assumed to be highly correlated with the survival rate and fertility of the M_1 population.

Radio-sensitivity test results following irradiation. Seedling growth or survival parameters measured are used to determine the LD_{50} or GR_{50} values for a few different seed crops. Table 1 shows data for plant height collected from three replications of gamma irradiation (^{60}Cobat, dose rate 140 Gy/min) of sorghum with doses 0, 75, 150, 300, 450 and 600 Gy. The values in Table 1 are plotted, either as percentage reduction in height or as percentage of the control, against the radiation doses applied.

$$\% \text{ mean} = [(\text{mean of treated}/\text{mean of control}) \times 100\%]$$

$$\% \text{ reduction} = [(\text{control} - \text{treated}/\text{control}) \times 100\%]$$

The values obtained for the two equations generates two graphs that are symmetrical and opposite in orientation (Fig. 7).

As the main outcome of the RST, the LD_{50}, GR_{50}, LD_{30} and GR_{30} can either be scored directly following the graph or by substituting in the slope equation of the linear trend line (Fig. 8). The doses used for the bulk treatment are usually estimated as LD_{50} or GR_{50} plus and minus 20%. Therefore, up to three dosages estimated from the RST can be applied for bulk irradiation, depending on the objective of the mutation breeding programme. In the example shown in Fig. 8 the dose to be applied

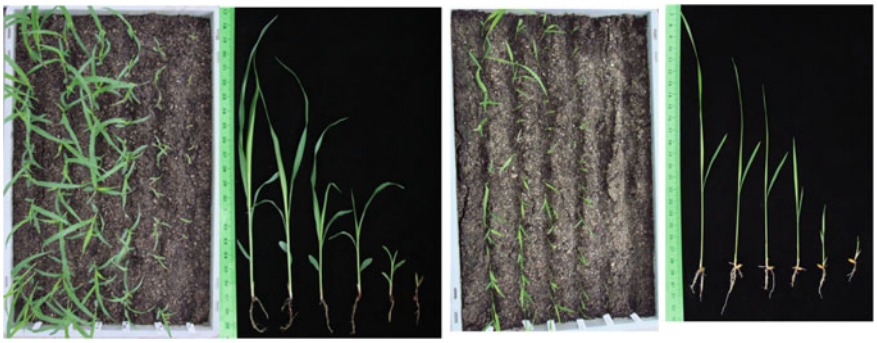

Fig. 6 Representative sets of photographs from radio-sensitivity testing in sorghum (**a** and **b**) and rice (**c** and **d**) seedlings two weeks from sowing of the gamma-ray irradiated M_1. Photos **a** and **c** are top views of single trays illustrating the trend in growth reduction and germination with increasing dose rate. Photos **b** and **d** are single representative seedlings per dose treatment showing the effects on growth after irradiation; from left to right: 0 (control), 75, 150, 300, 450 and 600 Gy

Table 1 Example of mean sorghum seedling height expressed as a percentage of the control and percentage reduction from the control after gamma irradiation of 20 seeds for each dose replicated three times

Applied dose (Gy)	Plant height (cm)	% height over control	% height reduction
0	24	100	0
75	23	95.8	4.2
150	18	70.8	28.2
300	13	54.2	45.8
450	7	29.2	70.8
600	3	12.5	87.5

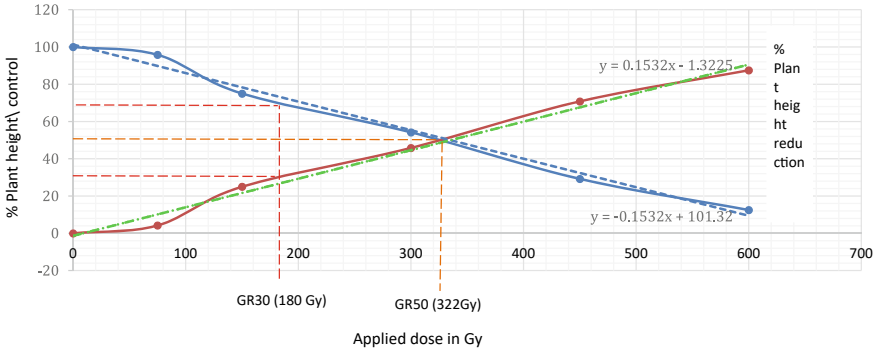

Fig. 7 Plotting of the data in Table 1 to show the effect of irradiation on sorghum seedling height expressed as percentage of non-irradiated (control) and percentage reduction in mean height. Red dots point to dose of GR30 (180 Gy) while green dots point to dose of GR50 (322 Gy)

for the bulk seed can be about 256, 322 and 384 and can be simplified to 260, 320 and 380 Gy if the choice is to apply around the GR50%. However, the same rule applies if the breeder chose lower doses around the GR30.

Relative biological effect of gamma-ray versus X-ray irradiation. Data was compiled from RST experiments on representative varieties of sorghum, rice and maize treated with in-house gamma-ray (^{60}Cobalt) and X-ray (RS2400) sources. This data is used to calculate and compare the relative biological effect (RBE) of gamma versus X-ray irradiation (Table 2). The data indicate that there are no significant differences between the two types of irradiation and the RBE ranged between 1.0 and 1.2. Generally, there is slightly greater damage with X-rays, which is most likely due to the different effects of each mutagen on DNA, with X-rays more likely to cause breakages leading to deletions (Fig. 8). As shown in Table 2, there is a very close trend in the RBE and the comparative reduction in germination and growth

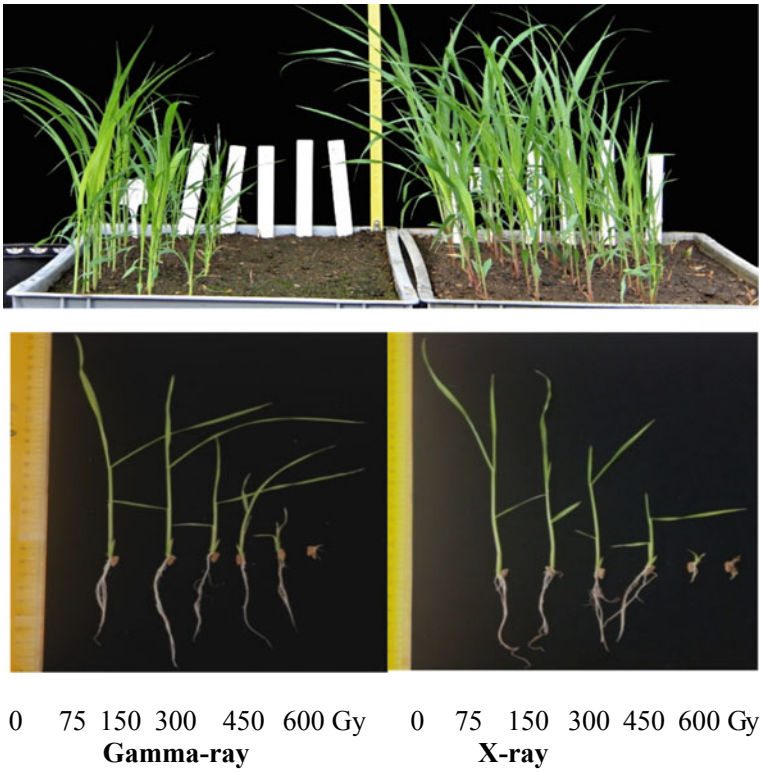

Fig. 8 Representative examples of photos comparing gamma-ray on two different sorghum varieties three weeks after sowing (top photos) and comparative growth of gamma versus X-ray irradiated rice seedlings (cv. Ashfard), 14 days after sowing (bottom photos)

rates. Different varieties of the same crop may have different sensitivity to irradiation and therefore it is recommended to run a RST before bulk treatment for each variety (Fig. 8).

Bulk treatment and handling of mutant populations. Initially the mutation breeding project starts with true-breeding highly homozygous seeds for treatment (M0). About 4000–6000 homogenous inbred seeds are prepared, cleaned and packed for irradiation. The proper amount of the M0 seeds depends on the dose, targeted trait and screening protocol. In the first mutant generation (M1), treated seeds are usually grown with spacing to maintain 2–3 tillers. They are protected from outcrossing to ensure self-pollination and each plant is harvested separately. In the second mutant generation (M2), seeds from each M1 plant are planted head-to-row with screening applied, selecting individual mutant plants separately (for qualitative traits). These are planted next to controls (untreated seeds) to verify identity of the mutant and exclude contaminations. In the third mutant generation (M3), seeds of selected mutants are planted for further selection and verification and to propagate selected individuals carrying the mutation. Selection for quantitative trait by head-row may start at M3. Measures are taken to ensure purity of the mutant selections by controlled self- or sib-pollinations. Once the mutant is isolated, the material can be handled in one of the three routes illustrated in Fig. 9. The direct route is through repeated cycles of selfing through M4–M6 generations (Fig. 10) to fix major quantitative genes and eliminate plants with inferior agronomic traits. Seed multiplication occurs at the M6–M7 generations concurrent with preliminary evaluation trials to select the best lines carrying the trait with good agronomic performance. Selections can then be

Table 2 Examples of GR30 and GR50 of some seed crops using a RS2400 X-ray irradiator and a gamma cell irradiator, and the relative biological effect (RBE) of X-ray irradiation over gamma-ray irradiation

Crop	Cultivar/genotype	Gamma-ray (Gy)		X-ray (Gy)		RBE (X/G-ray)	
		GR_{30}	GR_{50}	GR_{30}	GR_{50}	GR30	GR50
Sorghum	Arfa Gadamak	210	357	199	327	1.1	1.1
	Abu 70	234	362	210	313	1.1	1.2
	Tabat	189	332	185	311	1.0	1.1
	Aros Alramal	179	289	176	267	1.0	1.1
	Wad Ahmed	219	315	198	281	1.1	1.2
Rice	Ashfal	153	242	119	216	1.3	1.1
	Binadhan-14	254	353	243	346	1.0	1.1
	Sahel	185	278	167	245	1.1	1.1
	Kosti	221	313	189	294	1.2	1.1
	Umjar	237	331	201	312	1.2	1.1
Maize	Irat	219	357	204	328	1.1	1.1
	Hysen	227	343	213	324	1.1	1.1
	RXZ1	198	328	189	306	1.0	1.1

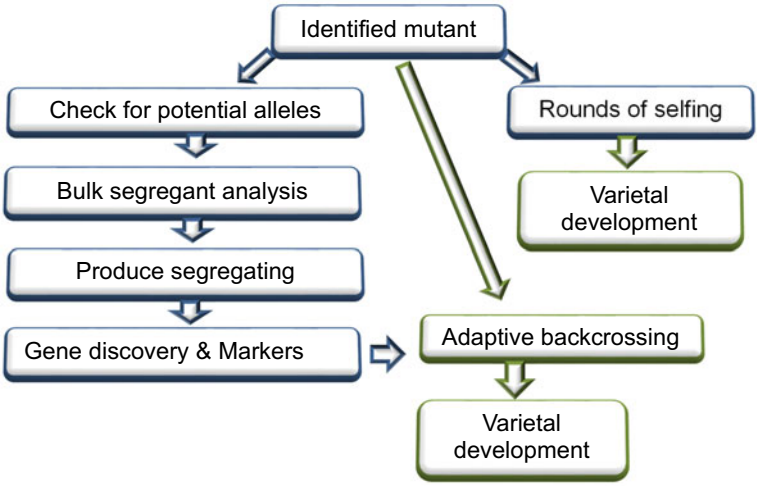

Fig. 9 Scheme illustrating the major three options for handling of the identified mutant towards development of mutant varieties. One of the options is to proceed for a few cycles of selection with selfing. A second option involves backcrossing the mutant to the unmutagenized precursor or other elite lines. A third option is the genetic analysis route for gene discovery and marker development

advanced to multi-location trials for the release and registration of the new mutant varieties (Mukhtar Ali Ghanim et al 2018).

The second route of mutant variety development is though backcrossing to the wildtype (unmutagenized) precursor or other elite lines and cultivars (BC1–BC5) (Fig. 9). Backcrossing can be to the wildtype parent to restore fitness from effects of random mutations in genomic regions outside those controlling the target trait. One or two backcrossing cycles might be sufficient to separate the induced desirable mutant trait from the undesirable background mutations. Improved lines resulting from mutagenesis can then proceed to registration as a new mutant variety.

The third route is to advance the isolated mutants to genetic analysis to understand inheritance of the induced trait and test for allelism if more than one mutant is isolated. Crossing is usually performed between the mutant and wildtype parent or an elite line with contrasting phenotype. Following the segregation in F2 and F3 families will enable inheritance studies. Bulked segregant analysis and comparative analysis of whole genome sequencing of the contrasting bulks can lead to discovery of the mutated genes and further development of molecular markers (see Sect. Conclusion Chapter "*Striga* as a Constraint to Cereal Production in Sub-Saharan Africa and the Role of Host Plant Resistance" for protocols). Molecular markers for the induced trait will feed subsequent backcrossing to transfer the trait to other varieties (Fig. 9).

There are several technologies which increase efficiency and accelerate the course of these three routes of handling mutant populations enabling fast delivery of the mutant varieties. These are described in Sect. Conclusion of this book.

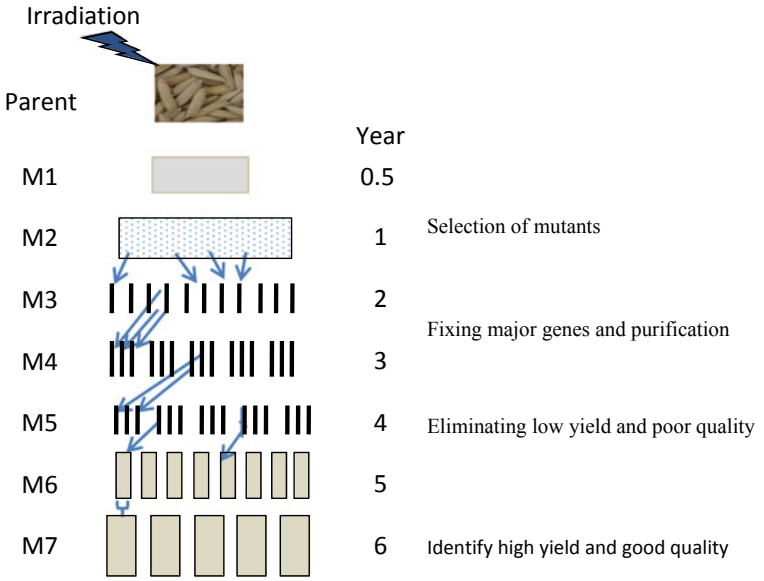

Fig. 10 Schematic drawing showing steps for the direct selection of the targeted mutant trait in M2/M3 and consecutive selfing and selection to better plants with good agronomic characteristics plus the targeted trait and fix them in stable lines in the M4–M6 generations to be advanced for multi-location evaluation for official release and registration

Conclusion

Simple and easy-to-follow seed irradiation protocols have been developed at the PBGL and are presented here for mutation induction in seed crops with examples of treatment of the targeted cereal crops (sorghum, rice and maize) using both gamma and X-ray as the most widely used physical mutagens. The GR_{30} and GR_{50} values for some seed crops (Table 2) are also presented, based on the data collected from radio-sensitivity testing during protocols development. These data may be used as a guide for mutation induction of bulked seed samples. The results presented here may vary depending on the type and characteristics of the irradiator used and the actual experimental conditions. An overview guide is presented for development and handling of mutant populations. The chapter serves as an introduction to the main chapters of the book on screening and efficiency enhancing technologies for development of resistance to *Striga* in the major cereal crops.

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Phenotyping to Identify Mutant Genotypes with Resistance to *Striga*

Screening for Resistance to *Striga Hermonthica* in Mutagenized Sorghum and Upland Rice in Burkina Faso

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Abstract *Striga hermonthica* is a major production constraint to sorghum and upland rice in Burkina Faso due to poorly structured and nutrient deficient soils, unreliable rainfall and high temperatures that limit the cropping season. A mutation breeding project using gamma rays as the mutagen was undertaken to improve *Striga* resistance in these two cereal crops in farmer preferred varieties adapted to the unique challenges of Burkinabe agriculture. This chapter describes the screening protocols used to select sorghum and upland rice mutants with improved productivity over unmutagenized lines.

Keywords Mutation breeding · *Striga hermonthica* · Resistance · Tolerance · Sorghum · Upland rice · Burkina Faso

Introduction

Burkina Faso is a Sahelian country with a sub-tropical climate characterized by a transition zone between the Sahel in the North (average annual rainfall of 350 mm) and the Sudanian zone in the South (average annual rainfall 1000–1200 mm). Its rainy season lasts four to five months (May–September) which is the main agricultural production period (Burkina Faso MAWF 2011). Cereal production in Burkina Faso is primarily subsistence-based and rainfed and therefore vulnerable to climate change, especially in the North where higher temperatures limit the cropping cycle duration. More than 85% of the working population is involved in agriculture, which

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contributes nearly a third of gross domestic product as well as accounting for more than 85% of export earnings (Burkina Faso MAWF 2011). Cereals are the main food resource for the population, with sorghum at the top (27% share of agricultural production) followed by pearl millet (25%), maize (8%) and rice (1%) (IFPRI 2017). These cereal crops are predominantly (99%) grown on rain fed lands.

Burkina Faso is the third largest producer of sorghum in Africa (behind Nigeria and Sudan) with an average yield of about 1000 kg/ha (Mundia et al. 2019). Sorghum is mainly grown in the Guinea-savannah and Sudano-savannah regions in areas receiving 700–900 mm annual rainfall. Total national sorghum production area is about 1.4 M ha (IFPRI 2017). White sorghums are preferred for food and therefore account for three quarters of national production. The rest is red sorghum, used primarily to brew the local beer, *dolo* and only used for food in times of food grain production shortfalls (Mundia et al. 2019). Both types are predominantly traditional cultivars since they are most adapted to the major agricultural constraints of the country, erratic rainfall and poor soils. Soils in Burkina Faso tend to be highly weathered with poor physical structure, low contents of active clay and organic matter, and low nutrient stores, imposing severe constraints to crop yields (Korodjouma 2022). It is precisely these conditions that make sorghum production vulnerable to *Striga*. *Striga hermonthica* occurs in almost all sorghum production areas of the country (Boussim et al. 2011).

Upland rice, for the same reasons, is also highly vulnerable to *S. hermonthica*. Total rice production in Burkina Faso is much less than sorghum, having only a 1% share of national cereal production with a 49,000 ha harvest area (IFPRI 2017). As in other African countries, Burkina Faso's rice consumption is constantly rising while national production barely covers 46% of the population's needs (Barro et al. 2021). Rice imports nearly tripled in the ten years between 1985 (137,185 t) and 2006 (305,180 t) (Burkina Faso MAWF 2011). To reduce dependence on imports, the Burkinabe government follows a National Rice Development Strategy which includes expanding cultivation of upland rice. Rice is produced by one of three cropping systems in Burkina Faso: irrigated, lowland and rainfed upland. Currently, rainfed upland rice accounts for only 10% of the rice land area (so only about 5000 ha) and 5% of national rice production (Barro et al. 2021). However, it could contribute more to national production, particularly if it can be slotted into the rotation systems of the cotton-growing regions in the wetter south. Being less resilient to drought than sorghum, upland rice is adapted to just those regions of Burkina Faso where the annual precipitation reaches or surpasses 800 mm (Burkina Faso MAWF 2011).

Striga resistance and tolerance is critical for sustained rice and sorghum production in Burkina Faso due to prevalent low rainfall, sandy and low fertility soils in low input agriculture which favors *S. hermonthica* (Boussim et al. 2011). Introducing *Striga* resistance into varieties already adapted to the unique challenges of Burkinabe environments and farmer preferences through mutation breeding offers durable protection (Nikièma et al. 2020). The protocols described in this chapter resulted from attempts to introduce *Striga* resistance through gamma irradiation into several Burkinabe sorghum and rice cultivars with susceptibility to the parasitic weed.

Protocols

Collecting Striga *seeds.* Green mature capsules of *Striga* plants without galls are harvested and air dried on large sheets of absorbent paper in the shade at room temperature (25–30 °C) for about 30 days (Fig. 1). Before drying, the top flowers must be removed and samples should be also shaken to remove any soil clods and other debris. When they are drying, the capsules open and release seeds on the absorbent paper. At the end of the drying process, the seeds recovered directly from the paper are considered as first quality while those that will be recovered on another paper after a light threshing are of second quality. Both batches are sieved using 315, 200 and 180 μm mesh sieves, successively. Seeds harvested at the end of the rainy season are dormant and can only be used in experiments after at least 5–6 months to allow the breakage of seed dormancy. Seeds must be stored in cloth containers (plastic and glass should be avoided for long storage) at room temperature or higher (ideally 30 °C).

Determining germination capacity of Striga *seeds.* Before using a new batch of *S. hermonthica* seeds, it is important to know their germinability. Since *Striga* seed require a period of conditioning under moist conditions for 12–14 days before they will germinate, they need to be surface sterilized and handled aseptically during the conditioning period before their response to germination stimulants is performed.

Under a laminar flow hood, place 0.5 g of *Striga* seeds in a 1.5 mL Eppendorf tube (this amount is sufficient for four 12-well plates). Add 70% ethanol to the tube containing the seeds and shake or vortex occasionally for three minutes. Remove the alcohol with a sterile pipet. Rinse once with sterile de-ionized distilled water (ddH_2O). Add 1 mL 3% bleach solution and shake vigorously or vortex until a brownish green color is obtained and allow to sit for five minutes. Shake again and let stand for an additional 2 min. Rinse with sterile ddH_2O at least five times until the rinse solution (sterile water) becomes clear. These multiple washes ensure removal

Fig. 1 Air drying of *Striga hermonthica* inflorescences (flowering stems) with capsules containing the seeds; **a** fresh inflorescences; **b** dried inflorescences

of sterilizing agents, dust, light seeds and plant debris. After removing the last water wash, the seeds remaining at the bottom of the tube should be clear of impurities.

The cleaned seeds may be conditioned in a multi-well plate or in a Petri dish. In both cases, sterile filter papers and plates must be used and transfers done under a laminar flow hood to ensure the seeds are not overtaken by mold or bacteria during the warm wet conditioning period.

Conditioning Striga *seed on a multi-well plate*. For this protocol, a sterile 12-well plate is used but 24- and 48-well plates can also be used, adjusting the filter paper circle size to fit the individual cells and volume of ddH_2O to saturate the glass microfiber discs in each well. The filter papers are sterilized beforehand by autoclaving. Multi-well plates can be purchased pre-sterilized. Aim for a density of 25 *Striga* seeds per cm^2 of glass fiber.

Using sterile forceps under a laminar flow hood, place one sterile 20 mm glass microfiber filter paper disc (Whatman GF/A) per well of a 12-well plate. Suspend surface sterilized *S. hermonthica* seeds in sterile ddH_2O. Using a single channel pipettor with sterile tips, take up suspended seeds in 50 μL and distribute *Striga* seeds on each filter to deliver 50–60 *Striga* seeds. Add 300 μL of additional sterile ddH_2O to each well. The total volume in each well is about 350 μL. The filter paper should be thoroughly wetted. Close the multi-well plate with its cover. Incubate them in the dark at 27–30 °C for 12–14 days.

Conditioning Striga *seed on a Petri dish*. Working in a laminar flow hood, line the bottom of a sterile 100 mm Petri dish with two sterile 90 mm Whatman No.1 filter paper circles.

Distribute 25–30 glass microfiber filter paper discs (Whatman GF/A) on the larger filter paper circles in the plate. Wet with 3 mL of sterile ddH_2O. With the aid of a pipettor with sterile tips, place 25–30 *Striga* seeds on each of the 11 mm discs. Add an additional 1 mL of sterile ddH_2O to the Petri dish, placing it on the directly on the backing paper circle to avoid dispersing the seed on the smaller discs. The filters should be wet but not under water. Add more or remove water with a pipette in contact with the background paper as needed. Seal the Petri dish with Parafilm M® barrier film and then wrap the plate with aluminum foil to exclude light. A few Petri dishes can be stacked together before wrapping if more *Striga* seed needs to be conditioned. Incubate in the dark at 27–30 °C for 12–14 days (Fig. 2).

Stimulating Striga *seed germination*. After the two week conditioning period, the *Striga* seeds can be germinated in the presence of low concentrations of strigolactones, either synthetic (GR24) or natural from host roots. Working under a laminar flow hood, add 30 μL of a 10 ppm GR24 solution to each well if the *Striga* was conditioned in a 12-well plate. Add an additional 270 μL of sterile ddH_2O to each cell of the plate. Replace the plate lid and slightly shake briefly with a horizontal movement back-and-forth. Incubate at 27–30 °C, in the dark for at least 48–72 h.

To *Striga* seeds conditioned in Petri dishes, add 3 mL of a 1 ppm GR24 solution to the plate applying directly onto the background paper. Seal the dish with Parafilm and wrap in aluminum foil. Incubate at 30 °C, in the dark for at least 48–72 h (Fig. 2).

If GR24 is unavailable, the cut-root technique described by Traoré et al. (2011) may be used as an alternative. This technique is based on the use of cereal root

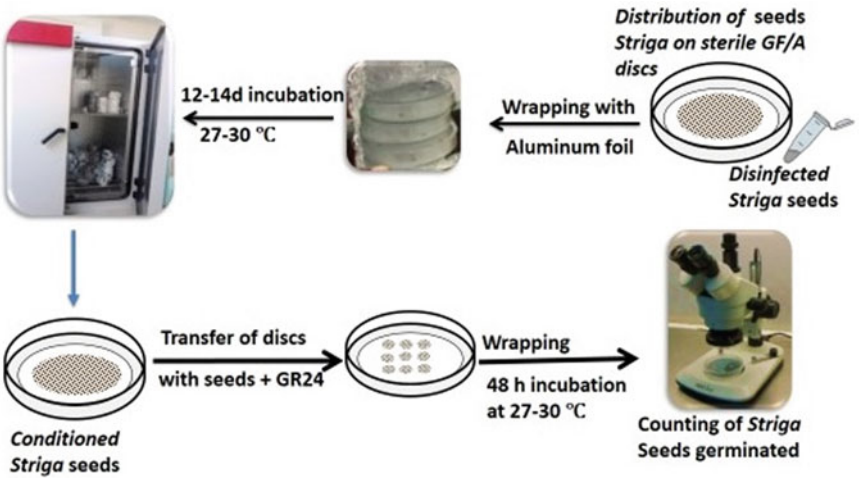

Fig. 2 Illustration of the main steps in assessment of germination capacity of *Striga* seeds using Petri dishes

pieces. In the assessment of *Striga* germination rate, the root of a known *Striga*-sensitive variety is used. For example in sorghum, 14-day old roots are harvested from seedlings grown in pot conditions, washed roughly with water and then cut up in pieces. One gram of the freshly cut sorghum root pieces are then placed in an aluminum foil ring (1.7 cm diameter) to stimulate *Striga* seed germination. Glass microfiber filter discs (Whatman GF/A, 8 mm) carrying conditioned *Striga* seeds are transferred into sterile Petri dishes (9 cm) lined with a double layer of Whatman No.1 filter papers. Discs with seeds are arranged in 4–5 lines (5 discs per radius) around the aluminum foil ring (Fig. 3). The root pieces in the ring are watered with 3 mL of sterile ddH_2O to enable the diffusion of root exudates. The entire root cut technique is illustrated in Fig. 4.

Determining Striga *seed germination rate.* Two to three days after the exposure to germination stimulants (GR24 or sorghum roots), position the plate under a binocular microscope to count both the number of germinated seeds and total number of *Striga* seeds on each disc (Fig. 5). From these counts, calculate the percentage of germination (= number of germinated seeds/total number of seeds × 100). Average these values from all discs to determine the germination rate of that particular batch of *Striga* seed.

Screening of cereal mutants for resistance to Striga hermonthica *under field conditions.* Field screening for *Striga*-resistance can start at the M2 generation or if seed is limited, this can be delayed until the M3. For field screening, a minimum of 10,000 M2 plants are screened. As an initial screen following gamma ray mutagenesis, very large populations (≥ 10,000 plants) of the early generations (M2–M3) in which recessive mutations leading to *Striga* resistance may be expressed are evaluated in field comparisons to the unmutagenized *Striga* susceptible progenitor line. It is best to start with at least 3–6000 gamma irradiated M1 seeds in order to obtain a sufficient

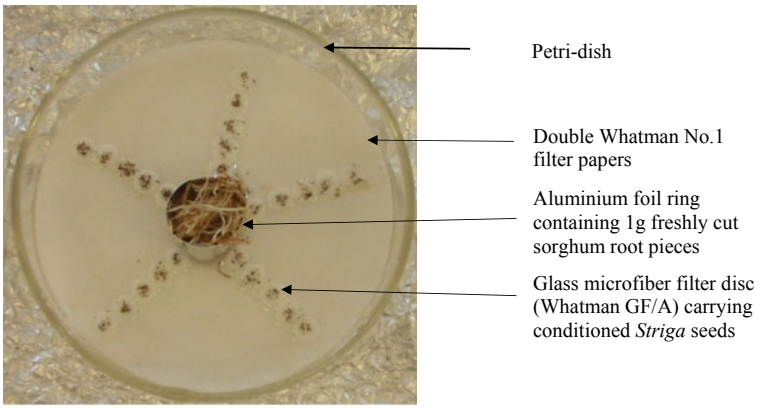

Fig. 3 Representative picture of root-cut technique

segregating M2 population in the range of 10,000 or more to enhance the probability of selecting a targeted mutant. Individual plants should be separated enough to distinguish them from their neighbors. The main criterion in this initial screen is whether an individual mutant plant (derivative of mutagenized seed after one or two generations of self-pollination) appears to be less affected by *Striga* according to the observations taken (e.g. fewer and/or smaller *Striga* emerged around it) relative to the unmutagenized check (Fig. 6). These initial tests concern large populations whose aim is to detect putative resistant mutants. Putative mutants can be advanced by self-pollination to M3 or M4 to confirm the heritability and stability of the *Striga*-resistance trait in subsequent trials.

Field trials should be conducted on historically *Striga*-free ground if available. The plots should be cleared of bushes and trees and their stumps, plowed to a depth of 10–15 cm, breaking up clods and leveling the ground as much as possible. The field should be relatively homogeneous and pre-treated with pesticide to clear termites. The planting area should be away from any tall vegetation that would shade any parts during the day.

For sorghum, the row length should be at least 8 m with 1 m between rows and 0.8 m between plants (Fig. 7). In each planting hole of 5 cm depth, spread approximately 5000 *Striga* seeds of recently tested good germinability. The experimental layout of plots is an "augmented design", so for each replication, plant the control (unmutagenized parent of the same variety as mutants) on the first row and then repeat after every ten rows of mutant lineages. Label each row indicating at least the name of mutant lineage or parent and the replication number.

For upland rice, the row length should be at least 5 m with 0.5 m spacing between rows and 0.5 m between planting hills. Spread 5000 germinable *Striga* seeds 5 cm deep in each of the hole where an upland rice seed is planted. The experimental set up is an "augmented design", so for each replication, plant the control (unmutagenized parent line of the mutant) on the first row and then repeat it after every ten rows of

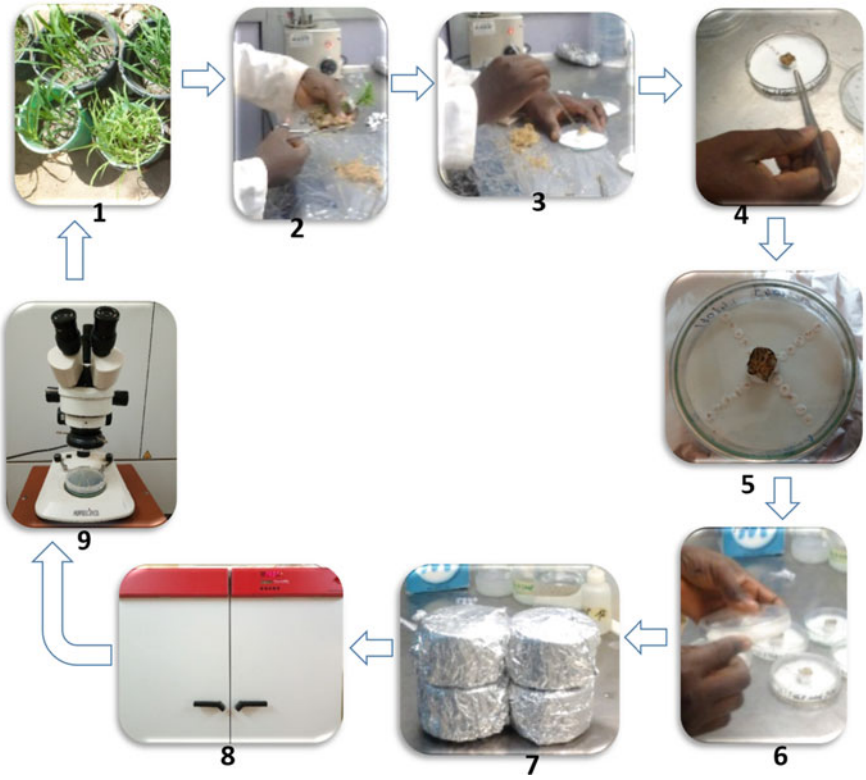

Fig. 4 Illustration of the main steps in carrying out the "cut-root technique" for the stimulation of *Striga* seed germination. (1) Sorghum seedlings are grown for 14 days in pots; (2) cutting of 14-day old sorghum roots into small (< 1 cm) pieces; (3) weighing and inserting 1 g root fragments into the aluminum foil ring; (4) arranging glass fiber discs with conditioned *Striga* seeds around the ring; (5) watering of root-cuts with 3 mL of sterile distilled water; (6) sealing the Petri dish with parafilm; (7) wrapping Petri dishes with aluminum foil; (8) incubating at 28–30 °C for 48–72 h; (9) counting germinated *Striga* seeds under a binocular microscope

mutant lineages. Label each row minimally with the name of mutant or parent and the replication number.

Management of screening plots include mild fertilization, weeding of all but *Striga*, and pest control. Fertilizers should only be applied when the soil is too poor to support cereal crops to modest seed production and doses should be minimal since poor nutrition stimulates *Striga* infection. Host plants should be treated with appropriate pesticides to mitigate pest attacks (insect pest like fall army warm, diseases caused by fungi or bacteria, etc.). Weed the plots twice with hand hoes at the soil surface at two weeks and again at four weeks after sowing, before *Striga* emerge. Any weeding after five weeks needs to be by hand-pulling to avoid disturbing any *Striga* which may emerge anytime after 35 days. Remove all weeds other than *Striga* from plots. The use of herbicide should be avoided from land preparation to harvest.

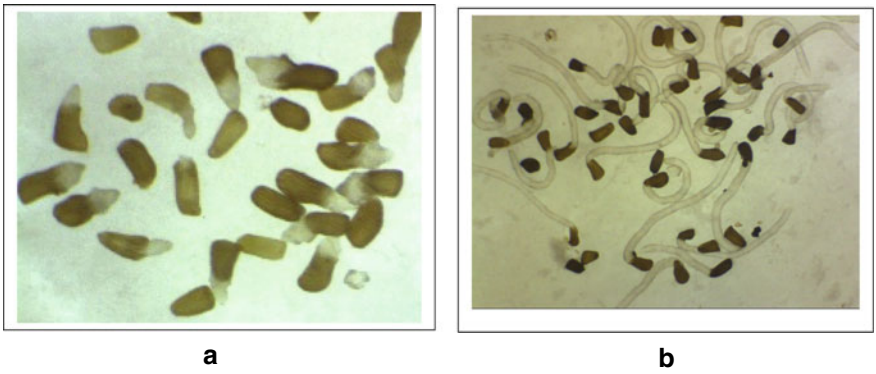

Fig. 5 Germinated *Striga* seeds after 48 h (**a**) and 72 h (**b**) after exposure to GR24 or sorghum roots

Fig. 6 Individual sorghum plants in *Striga hermonthica* infested plots late in the initial screening field trial. Putative mutants selected for advanced trials have visibly fewer emerged *Striga* around them (arrow) than unmutagenized checks

Fig. 7 Preparation of field plots marked for planting sorghum and *Striga hermonthica*

Screening in pots under screen-house conditions. Selfed seed from putative *Striga* resistant mutants selected from field M2 or M3 field plots can be verified either in subsequent field plots consisting of multiple progeny of the selected individual or in pots in a screen-house. Screen-house screening is intended to verify, confirm and characterize putative mutants selected under field conditions.

Use plastic pots of adequate size to support plants to maturity (for sorghum, ~ 20 L, e.g., 26 cm × 28 cm) with drainage holes filled with a 2:1 (v/v) mixture of soil and fine sand (Fig. 8). For artificial infestation of pots with *Striga* seeds, 1 kg of sieved sand is roughly mixed with 50 g of *Striga* seeds of which germination capacity is more than 70%. Layer the soil in the pots such that the bulk in the bottom is the 2:1 sand/soil mixture (~ 15 kg). Upon this add a mixture of (~ 3 kg) of the same soil mixture into which was mixed 10 g of the sand/*Striga* seed. The top layer consists of the same 2:1 sand/soil mixture put in the bottom (~ 2 kg). For upland rice, prepare 12 L pots (e.g., 28 cm × 18 cm), adjusting the soil volumes to achieve the same layers as illustrated for the sorghum screening pots in Fig. 8. This technique aims at an infestation rate of approximately 5000 *S. hermonthica* seeds per pot (Marley et al. 1999). Do not plant the cereal seeds yet. When all pots required for the screening are filled with soil substrate, transport them to a screen-house and water to field capacity. Keep them regularly watered so that the soil remains wet to allow conditioning of *Striga* seeds for 12–14 days.

The rice or sorghum can be prepared for sowing after the *Striga* has been allowed to condition in the screen-house pots for at least 12 days. Before sowing cereal seeds

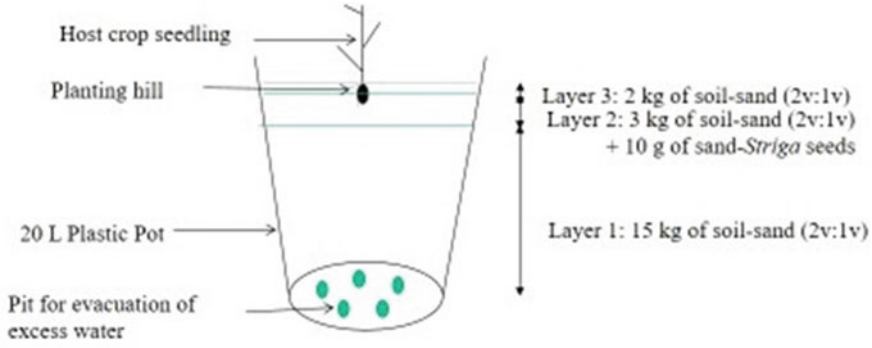

Fig. 8 Schematic representation of screening pot (note volume of soil and *Striga* seed mixture should be adjusted to the size of the pot used)

to be tested, surface-sterilize them with 70% ethanol for 3 min followed by 20% bleach for 5 min. Place these surface-sterilized seeds on sterilized wet filter paper in a Petri dish and incubate at 25–30 °C for 24 h. When the sorghum or rice seeds begin to germinate, with visibly emerged radicles, carefully sow then with forceps in a pre-excavated hole at a depth of 2–3 cm in the pots containing the conditioned *Striga* seeds with a single plant in each pot. Use 3–6 pots as replications for each mutant lineage being tested including positive (*Striga* infested pot + unmutagenized counterpart) and negative (uninfested pot) controls, the replication number will depend on the number of mutants to screen and the available space in the screen-house. Label each pot to indicate the name of mutant, replication number, etc. Keep pots minimally watered to support host plant growth, but not so wet as during the *Striga* conditioning period. Also maintain temperature and light and supply fertilizer sparingly as needed to support normal host plant growth. Apply pesticides or fungicides only if needed to mitigate any health threatening pests during the screening. Use the *Striga*-free control pots as indicators to guide management.

Selection of putative Striga *resistant mutants: Observations and records.* Detailed selection criteria are impractical in the early generations after mutagenesis (M2 or M3) when screening for possible *Striga* resistance gained through mutation. We used the criterion of fewer emerged *Striga* plants around M2 and M3 individual sorghum plants in the field screening protocol described earlier for 10,000 + plants (Fig. 6). From this screen, putative resistant mutant individual plants were self-pollinated to obtain M4 families of which multiple individuals from putative resistant selections could be more closely examined under *Striga* infestations in field or pots. By the M4, the selected trait (e.g., fewer emerged *Striga*), in so much as it is due to a specific genetic change, is generally fixed, and can therefore be characterized in terms of its impact on the parasitic relationship with *Striga*. It may be affected by other background mutations in the lineage that influence general plant fitness, but these may be sorted out through backcrossing to the original (unmutagenized) line

or through examining multiple plants in advanced generations tracing back to the particular M2 selected individual from which they were derived.

To characterize actual *Striga* resistance gained through mutagenesis, multiple variables may be measured in field and pot trials beyond the initial screen when population size was prohibitive. These include measurements like the time in days from sowing (DAS) to the first *Striga* emergence (Fig. 9a), *Striga* plant number emerged at 70, 90 DAS (Figs. 9b, 10 and 11) and at harvest, *Striga* plant death at 70, 90 DAS and at harvest and a *Striga* plant vigor score at 70, 90 DAS and at harvest (Table 1 and Fig. 12). These measures are compared to *Striga* in infested plots or pots of the original unmutagenized line. They indicate resistance if parasites are reduced in number, slower to emerge, prematurely die or are smaller relative to those on control plants. Resistance therefore depends on *Striga* performance, which is reflective of host plant support of their sustenance and growth.

Striga tolerance, in contrast, is measured by host plant performance under infestation. *Striga* notoriously negatively affects host plant growth. A host plant that succumbs to *Striga*'s negative effects is called sensitive. A *Striga* tolerant plant performs equally or nearly as well whether or not it is parasitized. Gained *Striga* tolerance, which will likely be influenced by reduced parasitism (resistance), may

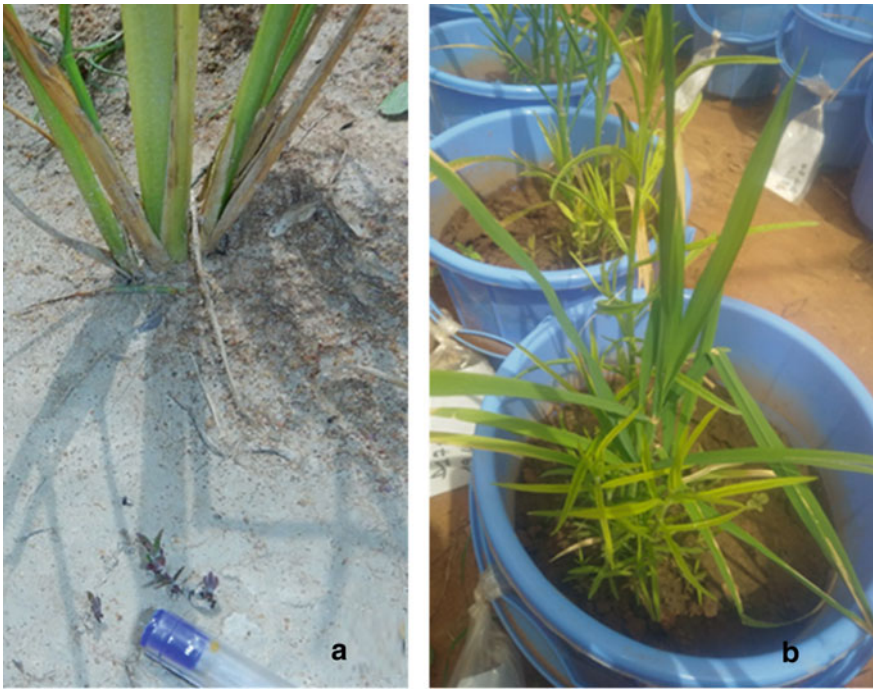

Fig. 9 *Striga hermonthica* plants at first emergence in field (**a**) and number of emerged *Striga* at 70 DAS in pot (**b**) conditions

Fig. 10 *Striga*-infested hills of sorghum plants at 90 days after the sowing

Fig. 11 *Striga*-infested hills of upland rice plants at 70 days after the sowing

Table 1 Scale score of vigor of *Striga hermonthica* plants

Score	Height of *Striga* plant (cm)	And/Or	Branch number of *Striga* plant
1	0		No *Striga* emerged
2	≤ 5		No branches
3	6–20		1–5
4	21–30		6–9
5	≥ 31		≥ 10

also be measured in these trials by comparing host plant performance with uninfested (*Striga*-free) plots or pots of the same mutant lineage by *Striga* damage scores at 70, 90 DAS and at harvest and through yield components of the host crop like biomass and grain. These must be compared to uninfested plants grown under the same conditions in the trial to indicate true tolerance.

Striga damage can be scored by the percent of bleached or burned leaves, a typical symptom of *Striga* infection. This value is calculated by dividing the number of burned leaves by the total number of leaves and multiplying that value by 100. A more detailed scale (from 1 to 9) considering leaf symptoms due to *Striga* infection described by Kim (1994) can be used that grades a host plant as normal with no visible symptoms of reduced growth or verdancy (1) through increasing stages of severity to complete demise (9) as that shown in Fig. 13.

Verification of putative mutants in pots. Generally, pot screening aims at verifying putative mutant traits observed by the field screening. Because the soil in the pots can be dumped at harvest, attached *Striga* can be separated from host roots after washing away the soil. This allows one to determine the biomass of all attached parasites, as well as obtaining host root and shoot weights and the root:shoot ratio of infested host plants. These additional measures can be used to further define both resistance and tolerance. Resistance may be expressed as reduced *Striga* biomass on the mutant potted plants (Fig. 14) relative to those on unmutagenized control potted plants. Tolerance expressed as milder reductions in host plant shoot height and weight and root weight can be measured by comparing plants in *Striga* infested pots to plants of the same mutant grown in uninfested pots (Fig. 15). *Striga* generally increases host plant root:shoot ratio by stimulating root growth and reducing shoot growth. *Striga* may have less influence on root:shoot ratio in tolerant plants. From such tolerance parameters, indices may be calculated by dividing the measured value from infested pots (e.g. host plant height) by that measured on the same genotype in uninfested pots. Such a value is unfortunately sometimes called a "resistance index", though it is more precisely an indicator of tolerance. Tolerance is usually influenced by resistance since a resistant plant has fewer parasites and therefore less likely to be affected by *Striga*'s negative impact. Another common measure is percent host plant growth reduction (GR) determined by the equation $GR = \frac{(x-y)}{x} \times 100\%$, where x is an indicator of host plant growth in *Striga*-free pots (e.g., height. shoot or root weight) and y is the same parameter measured on the same genotype under *Striga* infestation. A similar yield reduction (YR) can similarly derived from grain yield substitutions

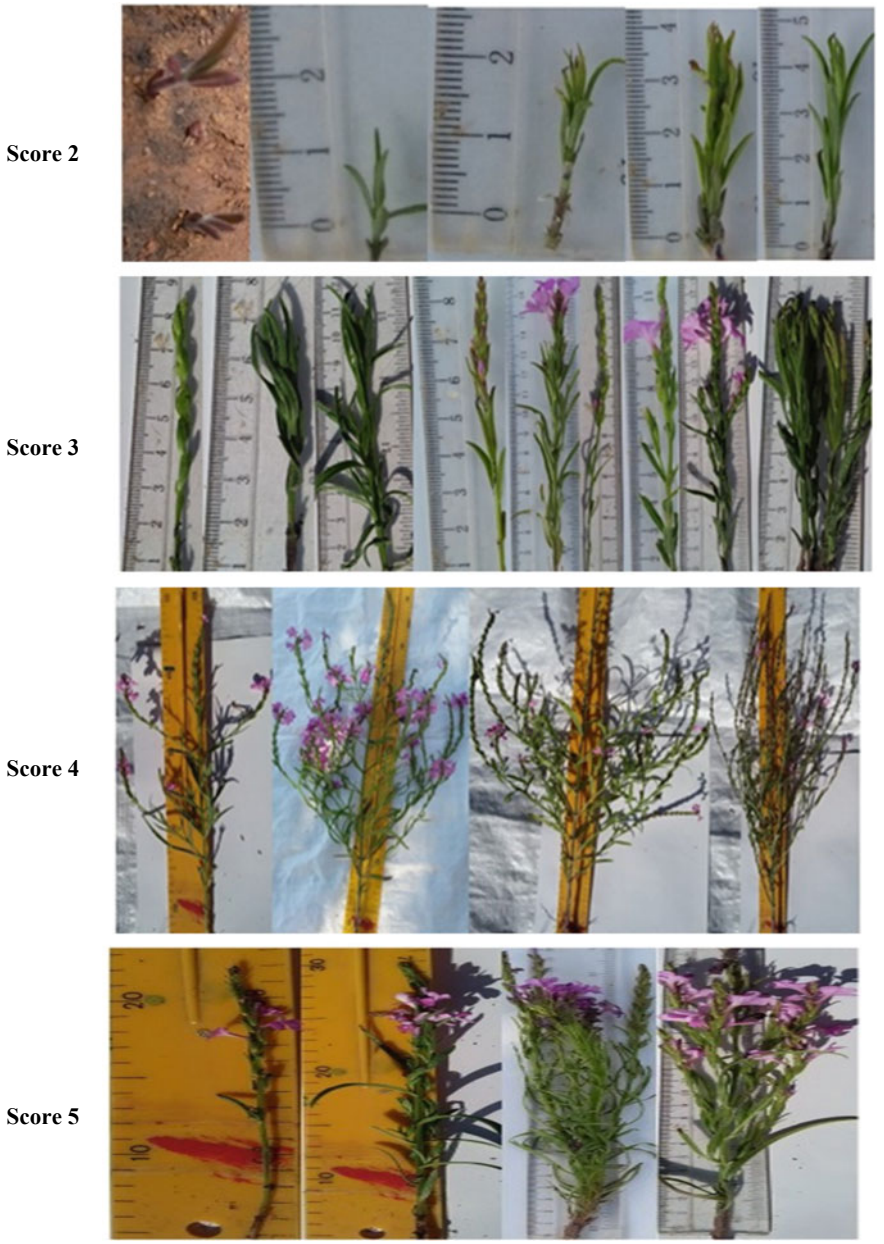

Fig. 12 Illustrative pictures of *Striga* plant vigor scores

Fig. 13 Premature death of a sorghum plant and no panicle formation due to *Striga* infection

in the formula. These measures, especially those involving underground plant parts, are prohibitively difficult in field grown trials.

Ranking of cereal mutants according to their reaction to Striga hermonthica. Putative *Striga* resistant mutants selected in early generation field screens are ultimately ranked based on additional field and pot testing of their progeny for further characterization. First, each mutant lineage was ranked as susceptible if they showed no significant improvement over their unmutagenized counterpart and dropped from further consideration. Those advanced to later generation trials were classified into one of four phenotypes: resistant, neutral, tolerant and sensitive/susceptible (Table 2). Resistant and tolerant lineages were advanced for further investigation which might include laboratory testing to determine the mechanism of resistance (as in Chapters "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum" and "Histological Analysis of *Striga* Infected Plants") and cultivar development.

Protocol validation in the case of mutagenized sorghum and upland rice in Burkina Faso. Application of these protocols to screen gamma irradiated sorghum and upland rice populations succeeded in selecting putative Burkinabe mutants resistant to *Striga hermonthica.* The field screening protocol was conducted on 6770 plants of M3

Fig. 14 Differences in the amount of *Striga* plant biomass influenced by sorghum genotype are obvious by harvest. Left and center panels are putative resistant mutants derived from gamma irradiated lineages, with the unmutagenized control on the right. *Striga* can be removed and weighed at harvest to determine total biomass supported by each host plant

Fig. 15 Various parameters like plant height, shoot biomass, root biomass, root:shoot ratio and leaf damage scores determined from potted host plants (pearl millet pictured here) can indicate tolerance to various degrees of *Striga* infestation by comparing those values with those of the same genotype grown in *Striga*-free pots (left). *Photo credit* Dale Hess

Table 2 Phenotypic classification of a mutant plant according to reaction to *Striga hermonthica*

Classification based on *Striga* reaction	*Striga* density 45–60 DAS	*Striga* density 90–120 DAS	Relative to unmutagenized counterpart	
			Striga biomass* at harvest	Yield components of host crop*
Resistant mutant	0–2 emerged *Striga*	≤ 5 emerged *Striga*	Highly reduced	Not affected
Neutral mutant (slightly improved over unmutagenized counterpart)	3–4 emerged *Striga*	6–15 emerged *Striga*	Slightly reduced	Affected
Tolerant mutant	≥ 5 emerged *Striga*	≥ 16 emerged *Striga*	Slightly higher	Not affected
Susceptible and sensitive mutant (no improvement over unmutagenized counterpart)	≥ 5 emerged *Striga*	≥ 16 emerged *Striga*	High	Highly affected

**Striga* plant biomass and host yield components (plant height, grain yield) were determined from potted plants in a screen-house

sorghum families, 3385 plants of M4 sorghum lineages and 3465 plants of M3 upland rice families at Kouaré research Station (11°95′03″ N and 0°30′58″ E) in the eastern Sudano-savannah region of Burkina Faso. The field soil, levelled to avoid localized ponding (Fig. 7) was sandy-loam, tropical, and ferruginous. During the crop growth, 362.8 mm of rainfall fell over 24 days, representing 43.5% of the annual rainfall of the year. *Striga* infected plants at crop harvest (≥ 3 emerged *Striga* plants per hill) varied from 95.8 to 97.6% for sorghum and 94.7–97% for upland rice within the mutant plants of the same variety. Mutant plants that induced 0–2 emerged *Striga* plants/hill or late emergence of ≤ 3 *Striga* plants/hill (at cereal grain filling stage) or the death of many nearby *Striga* were selected for advanced trials as putative *Striga*-resistant M3 lineages (were from the same M3 head row). These selected *Striga*-resistant lineages represented 1.8–2.7% of all mutant sorghum plants and 2–2.8% of mutant upland rice plants. Six hundred ninety-nine (M3/M4) and 221 (M4/M5) sorghum mutants and 105 M3 and 32 M4 rice mutants were advanced to further trials. The mutant lineages that continued to appear *Striga* resistant in subsequent field trials were advanced to pot screening. Significant results from screening of sorghum mutants in field and screen-house conditions are published (Nikièma et al. 2020). Screen-house screening confirmed the *Striga*-resistance of two upland rice mutants (Fig. 16).

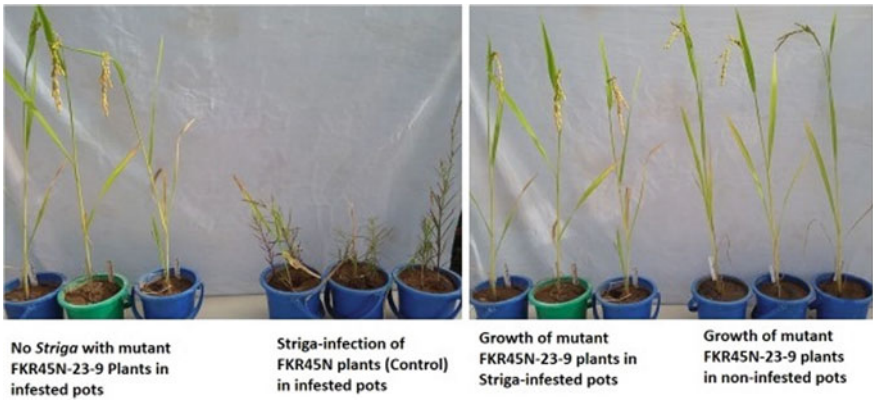

Fig. 16 Example of one *Striga* resistant upland rice mutant selected from gamma irradiated derivatives of a Burkinabe variety. (Left photo) *Striga* emergence in mutant FKR45N-23-9 pots (left) compared to the unmutagenized original line, FKR45N (right). (Right photo) plant growth of the mutant in *Striga* infested (left) compared to uninfested (right) pots

Conclusion

The protocols described in this chapter were successfully used to select *Striga* resistant mutants from gamma irradiated sorghum and upland rice adapted to the challenging environments of Burkina Faso. The narrow window of sorghum and upland rice cultivation in sub-Sahelian regions where soils are generally poor and rainfall scarce is quite vulnerable to *S. hermonthica.* Introducing *Striga* resistance through mutagenesis in cultivars adapted to these conditions can help to sustain sorghum and expand upland rice production in the country. Improvements to the field screening trials described in this chapter for *Striga* resistance would be to treat the seeds with pesticides before sowing to prevent losses to termites and birds and to plant just before a rain or to water hills if rain fails to ensure crop emergence. Despite these challenges, some promising *Striga* resistant mutants were selected in both sorghum and upland rice. Further characterization in both laboratory assays (Chapters "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum" and "*Striga* Germination Stimulant Analysis") and multi-location field trials will determine the ultimate value of this work and lead to cultivars that improve sorghum and upland rice production in Burkina Faso. Similar efforts are described in Chapter "Mutation Breeding for Resistance to *Striga* Hermonthica in Sorghum and Rice for Sustainable Food Production in Sudan" for sorghum and rice in Sudan and in Chapter "Phenotyping for Resistance to *Striga* Asiatica in Rice and Maize Mutant Populations in Madagascar" for maize and rice in Madagascar.

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Mutation Breeding for Resistance to *Striga Hermonthica* in Sorghum and Rice for Sustainable Food Production in Sudan

Ayman Abdel Maged Awad

Abstract *Striga* is the most limiting biotic constraint to sorghum and rice production in much of Sudan. This chapter describes protocols used to screen for *Striga* resistance among gamma irradiated elite sorghum and rice cultivars adapted to Sudanese agroecologies. The original cultivars show high yield potential but are *Striga* susceptible. The physical mutagenesis attempted to create genetic variants among these Sudanese cultivars with improved *Striga* resistance. Methods for collecting, cleaning, storing and testing germination of *S. hermonthica* seeds suitable for resistance screening are also described.

Keywords *Striga hermonthica* · Sorghum · Upland rice · Mutation breeding · Resistance

Introduction

In Sudan, sorghum is grown on over six million hectares, 30% of which are infested with *S. hermonthica* (Samejima et al. 2018). Although it has the most land devoted to sorghum production, a 17.3% global share, its global production share is only 7.8% (Pingali et al. 2020). Despite its versatility and economic importance in the livelihoods of millions of subsistence farmers, average sorghum productivity in Sudan is only about 700 kg/ha, less than half the global average (Pingali et al. 2020). This yield shortfall is mainly due to the use of traditional low yielding varieties, limited fertilizer use, low erratic rainfall and *Striga* infestation (Samejima et al. 2018). Increased use of higher yielding sorghum varieties with *Striga* resistance among subsistence farmers could substantially alleviate these dramatic productivity shortfalls.

Rice has become a staple food in many countries of Africa and constitutes a major part of the diet, particularly in urban areas. In Sudan rice is the fourth major

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food source, after sorghum, millet and wheat (Elhassan 2017). Although rice is mainly cultivated under irrigation in areas along the White Nile, the use of upland varieties expands the potential rice growing areas to 300,000 ha (Elhassan 2017). As in many African countries, rice demand exceeds production in Sudan requiring net import to meet demand at a huge cost in hard currency. Sudan consumed a total of 90,000 tonnes of rice in 2020 but produced only 35,000 tonnes (FAO 2021). The ability to grow upland rice could meet Sudan's domestic needs. As with Sudan's other major cereals, upland rice production is constrained by *S. hermonthica* (Samejima et al. 2018). Protecting rice yields from losses through cultivation of *Striga* resistant varieties could further ensure sufficient production to meet Sudan's domestic needs.

Although some *Striga* resistance and tolerance is known among both rice and sorghum cultivars (Dafaallah et al. 2019; Samejima et al. 2018), broadening genetic diversity underlying these traits through mutation breeding offers further and more durable protection. Introduction of resistance into otherwise agronomically promising but *Striga* susceptible cultivars could have a very positive impact on Sudan's cereal productivity. The protocols described herein are derived from an attempt to introduce *Striga* resistance through gamma irradiation into several improved Sudanese sorghum and rice cultivars with susceptibility to the parasitic weed.

Protocols

Striga *seed harvesting. Striga* seed is best harvested in late October in rain fed areas of Sudan after the capsules turn brown in color. *Striga hermonthica* flowers progressively from the bottom to top of the floral head (Fig. 1). When collecting seeds, it is best to cut only the upper part (floral heads) of the *Striga* plants (Fig. 2). This will lessen the amount of trash to be screened out later. A mature floral head is one on which all florets have completed flowering, with no visible flowers or only the upper flowers remaining (Fig. 3). Harvest only the mature heads with mature capsules (Fig. 4).

Although a single *S. hermonthica* plant may have multiple flowering branches and a single plant may produce several thousand seeds, the seeds are quite small (Fig. 4) so several plants must cut and collected to get an appreciable volume of seed suitable for pot and field studies. The harvested mature florets should be spread on a 4×4 m white polyethylene sheet to dry. Mix the *Striga* plants daily to avoid rotting and to ensure even drying. After 10–14 days of drying, tap the floral heads gently on the plastic sheet to release the seeds from the dried capsules.

After "threshing" all of the *Striga*, screen the material on the sheeting by passing it through sieves of 250 and 150 openings. Sieving helps remove most of the non-seed plant debris in the seed lot and makes subsequent infestations with the seed more accurate. In addition, the seed will store better and be less susceptible to fungal spoilage once this debris is removed. Most of the *Striga* seed will be collected on the 150 μm screen (Berner et al. 1997; Figs. 5 and 6).

Fig. 1 Flowering *Striga hermonthica* plants

Fig. 2 Mature *S. hermonthica* plants with seed capsules

Cleaning Striga *seeds*. The *Striga* seeds can be separated by weight from further impurities and cleaned in a 500 mL beaker containing 100 mL 70% ethanol, 50 mL 20% bleach (1% NaOCl) and 300 mL tap water. Add 50 g of the sieved *Striga* seeds into the beaker containing the liquid and stir it gently with a spoon for three minutes. Let the seeds rest in this solution for 20 min. Heavier particles, including viable

Fig. 3 A *Striga* floret nearly completed flowering

Fig. 4 Mature *Striga* capsules shedding seed

seeds, precipitate to the bottom of the beaker, while the lighter particles (empty seed coats, immature or bug eaten seeds, bits of capsules) will float on the surface of the liquid (Figs. 7 and 8).

Fig. 5 Threshed unsieved *S. hermonthica* seeds

Fig. 6 *Striga* seeds after sieving

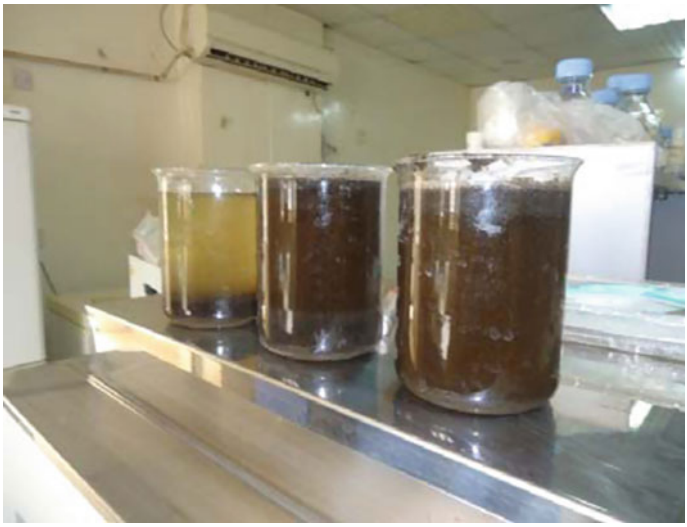

Fig. 7 Washing sieved *Striga* seeds

Fig. 8 Viable *Striga* seed settles to the bottom

Skim the debris (upper) layer off with a spoon then carefully pour off the cleaning solution into a waste container leaving the *Striga* seeds in the bottom of the beaker. Use a Pasteur pipet fitted with a bulb to suck away as much of the remaining liquid from the seeds as possible. Pressing the end of the pipet flat against the bottom of the beaker while the bulb is compressed and then slowly releasing the bulb will suck

Fig. 9 Washed *Striga* seeds spread on a blotter to dry

up the liquid without the seed. Add 50 mL tap water to wash the remaining seeds, stir together gently and let the seeds resettle for at least 10 min. Pour away the wash water to waste and suck away remaining liquid as before. Repeat the washes several times until the liquid above the settled seeds looks clear. Used distilled water for the last wash. After sucking away the remaining liquid in the final wash with the pipet, invert the beaker over a tray lined with white blotter paper. Tap the bottom of the beaker to expel the seeds onto the blotter paper. Use the spoon remove any seeds still in the beaker and spread the washed seeds evenly in a thin layer across the blotter. Allow them to air dry at room temperature overnight (Fig. 9).

Striga *seed storage*. Once the seeds are fully dry, they will not clump together and can be carefully poured or scooped from the paper into waterproof glass, plastic or metal jars or vials. The clean dried *Striga* seeds can be stored for years at room temperature in these storage containers as long as they remain fully dry (Fig. 10). Putting the containers in a desiccator helps to keep them dry. The stored seeds should be checked periodically for fungal and/or insect damage, especially in the first days and weeks after washing. If such damage is evident, resieve and rewash the seeds as before. Check the germination rate after a year of storage as described below and recheck annually or before each time they are used. They should have a germination rate of at least 30%.

Checking the Striga *seed germination. Striga* seed has an after-ripening requirement of several months to a year after they are collected. Be sure they have been properly cleaned and stored for at least 10 months before testing their germinability. Surface sterilize the *Striga* seeds by placing a small scoop of dried seed in a 25 mL flask. Add 10 mL 70% ethanol, move to a laminar flow hood and agitate the seeds

Fig. 10 Example containers for *Striga* seed storage

in the liquid with a sterile Pasteur pipet fitted with a bulb for 5 min. Carefully pour off the ethanol solution into a waste container after the seeds have settled. Use the Pasteur pipet to suck away as much of the remaining liquid from the seeds as possible. Pressing the end of the pipet flat against the bottom of the flask while the bulb is compressed and then slowly releasing the bulb will suck up the liquid without the seed. Add 10 mL sterile distilled water and agitate for 5 min with the pipet to wash the seeds. Remove the wash water from the flask as with the ethanol. Add 10 mL 20% bleach (1.05% NaOCl)/0.2% Tween® 20 (Sigma-Aldrich) to the flask of seeds and agitate with the pipet for 5 min. Remove the liquid as before. Wash three times with 10 mL sterile distilled water as before. Leave the flask open under the laminar flow hood after removing the final wash liquid to air dry overnight.

Conditioning the surface sterilized *Striga* seeds for a week in a 100 mm petri dish following these steps. Place a 90 mm Whatman #1 filter paper disc in the petri dish. Add 1 mL of sterile distilled water to the dish to wet the filter paper. Place twenty 15 mm Whatman #1 filter paper discs onto the larger wetted paper in the petri dish. Sprinkle 20–40 dried surface sterilized *Striga* seeds to each small disc with the aid of a small metal spatula previously wiped with 70% ethanol. Add an additional 1 mL water with a pipet onto the bottom paper, taking care not to wash the *Striga* seeds off the small discs. Close the petri dish and seal with parafilm. Place petri dish with seeds in a dark incubator set at 30 °C for one week.

After one week of conditioning, open the plate containing the *Striga* seeds under a laminar flow hood. Apply a total of 1 mL by pipet of a 10 ppm strigolactone germination stimulant (strigol or GR24) onto the 90 mm bottom paper in the petri dish taking care not to wash any *Striga* seeds from the 15 mm discs. Try to move the

pipet tip to different exposed areas of the bottom paper surrounding the smaller discs as the solution is delivered so that the germination stimulant solution is distributed evenly across the plate. Close the petri dish and seal with parafilm. Place the petri dish with seeds in a dark incubator set at 30 °C for 48 h. After the two days of exposure to striogolactones, count the germinated and total number of seeds on each of the 20 discs seeds under a stereomicroscope. Add the total number of germinated seeds from all 20 discs and divide by the total number of seeds (germinated and ungerminated) on all discs to determine the germination rate of the seedlot. If it is at least 30%, it may be used for infestations of pots or planting hills as described below.

Striga *seed inoculum preparation for infesting pots or planting hills.* Because *Striga* seeds are so small, soil infestations are most easily accomplished if the *Striga* seeds are mixed with a carrier material to increase volume. Sand is a good carrier, but it should be sieved so that only particles of the same size as *Striga* seed are used. A 250 or 150 μm sieve should be used for sieving the sand. To prepare uniform artificial *Striga* seed inoculum for infesting pots or planting hills, evenly mix 1 g of cleaned stored *S. hermonthica* seeds determined to have acceptable germinability with 2 kg of sieved sand. Add one full cap from a 2 L plastic bottle (approximately 10 cc) of *Striga* inoculum per planting hill of 5 cm deep and 5 cm wide. This should contain approximately 2000–2500 germinable *Striga* seeds.

Striga hermonthica infests all the major cereals grown in Sudan (sorghum, millet, rice and even wheat). There does seem to be, however, *Striga* strains specifically adapted to each host species (Dafaallah 2020). Therefore, resistance screening for sorghum should be done with a *Striga* seeds collected from a sorghum field and for rice with those collected from rice plots.

Field protocol followed for Striga *resistance screening of mutagenized sorghum.* Seeds of four Sudanese farmer-preferred cultivars with good yield potential were gamma irradiated with a ^{60}Cobalt source at the IAEA PBGL as described in Chapter "Physical Mutagenesis in Cereal Crops". Three doses, 100, 200 and 300 Gy, were chosen based on the radio-sensitivity experiments performed at PBGL. The mutant seed lots (M1) population was sent to Sudan and immediately grown in the field to generate the M2 seeds. About 1000 M2 plant seeds from each of the three doses of the four farmer preferred cultivars were screened under *Striga* infestation in rainfed field plots. Equivalent plots of unmutagenized seed were used as controls.

Sow the sorghum seeds into 10 m long row, with holes spacing of 20 cm within row and 80 cm between rows. Add one cap from a 2 L plastic bottle (≈ 10 cc) of *Striga* seed inoculum in each hole. Thin sorghum seedlings to one plant per hole ten days after sowing. Apply N fertilizer (urea) equivalent to 18 kg/ha around the plants four weeks after sowing. This delayed and suboptimal urea fertilizer rate should maintain sorghum growth but add sufficient nitrogen stress to favor *Striga* infection. Weed the plots of all but *Striga* by hand at 15, 30, 45, and 60 days after sowing. Count the number of emerged *Striga* around each sorghum plant beginning at two weeks after the appearance of the first *Striga* in the plots and repeat these counts three times at two week intervals.

Field protocol followed for Striga *resistance screening of mutagenized rice*. Seeds of the three upland rice cultivars, released for their commercial productivity in Sudan, were gamma irradiated with a ^{60}Cobalt source in doses of 100, 200 and 300 Gy at the IAEA PBGL in Austria as described in Chapter "Physical Mutagenesis in Cereal Crops". The irradiated M1 seeds and non-irradiated seeds (control) were sent back to Sudan for field testing. Approximately 600 irradiated seeds from each dose of each cultivar were sown individually in the field in order to raise M1 plants. Separate control plots consisting of 600 seeds of non-irradiated seeds of each cultivar were sown near for comparison of survival rates. Fitness of M1 plants were determined by comparing days to heading, days to maturity and grain yield to controls.

The *S. hermonthica* field screening experiment was conducted during the rainy season of 2017 (July–October) at the research farm of the White Nile research station at Kosti, Sudan. No supplemental irrigation was applied to the plots. M2 seeds of the three upland rice cultivars at the three different doses were sown along with seed from their unmutagenized parents in *Striga* sick plots to estimate whether mutagenesis improved *Striga* resistance (measured by number of emerged parasites per plot) in individual M2 plants relative to controls. Seeds were sown in a row trail, with spacing of 20 × 20 cm between rows and three seedlings per hill. Each experimental plot consisted of 20 rows. *Striga* inoculation was done as previously described for sorghum (10 cc *Striga*/sand mixture in each planting hole). Weeds other than *Striga* were removed by hand 15, 30, 45, and 60 days after sowing.

Individual M2 sorghum or rice plants around which no *Striga* emerged were advanced to M3 by self-pollination. Of the approximately 1000 M2 sorghum and 5400 M2 rice plant seeds from the three irradiation doses (100, 200 and 300 Gy) of farmer preferred cultivars field screened for *Striga* resistance, 46 sorghum and 62 rice lineages of promising putative *Striga* resistant mutants were selected. M3 seed from individual M2 selections were retested with *Striga* infestation in pots in a greenhouse.

Sorghum pot experiment. Fill each plastic pot (round, 20 cm diameter) with 2 kg of a mixture of field soil and sand (3:1, v/v). Dig a 5 × 5 cm hole in the middle of each pot and add to this one plastic bottle cap (10 cc) of *Striga* inoculum as described previously. Move pots to a greenhouse and water to keep the soil mixture damp for 7–10 days to condition the *Striga* seed. Re-excavate the 5 × 5 cm hole in the middle of each pot sow three sorghum seeds. Plant four pots of each M3 lineage. Continue to water as needed to keep soil visibly moist. Thin sorghum to one plant per pot at two weeks after sowing. Remove any weeds other than *Striga* by hand at 15, 30, 45, and 60 days after sowing. Count the number of emerged *Striga* plants at 45, 60 and 90 days after sowing.

Rice pot experiment. M3 seed of the rice mutant candidates were screened in the greenhouse similarly to sorghum, except that plastic bags of similar volume (2 kg) holding the same 3:1 soil/sand medium were substituted for rigid pots. Six of these bags were prepared per mutant lineage, four infested with the *Striga* seed inoculums and two without as an addition control to the unmutagenized counterpart of each tested lineage. Comparing host plant fitness parameters (height, weight, verdancy, grain yield) in infested pots with those growing without *Striga*, can give an indication

Fig. 11 Two rice M3 lineages from independent M2s selected from *Striga* free plants in infested field plots. Individual M3 plants (first four bags in each series) from both lineages remained *Striga* free in infested pot trials in contrast to their respective unmutagenized parent lines (fifth bag at right of each series)

of *Striga* tolerance. As with sorghum, sown M3 seeds were thinned to one rice plant per pack two weeks after sowing. Plants were watered at two day intervals throughout the experiment. *Striga* emergence counts were taken at 45, 60 and 90 days after sowing.

Quantification of gained Striga *resistance.* The *Striga* resistance of M3 potted plants advance from M2 field selections was quantified by plotting the average emergence count of the four plants per lineage over the three counting dates (45, 60 and 90 days). The area under these *Striga* progress curves were then compared to those of the respective unmutagenized progenitor lines (Gowda et al. 2021). Those showing an area less than half that of the original line were considered to have potentially gained *Striga* resistance through mutagenesis. Gained *Striga* tolerance over uninfested controls was not quantified. Of the 46 sorghum and 62 rice M2s advanced to pot studies, ten sorghum and 22 rice M3 lineages were selected as the best putative mutants for further characterization in laboratory assays, pots and field evaluations. Two of the rice selections in which no *Striga* emerged in the pot study are pictured in Fig. 11.

Conclusion

Striga hermonthica is a major contributor to low cereal productivity in Sudan, including sorghum and rice. Mutagenesis derived cultivars with good yield potential and *Striga* resistance can improve rice and sorghum production and contribute to national food security. From the protocols described in this chapter, ten putative *Striga* resistant mutants of sorghum and 22 of rice were identified in lineages from independent gamma irradiated events. These were selected from field screens of M2

populations and confirmed in derived M3 pot screens. Further agronomic characterization of these mutants to determine yield under *Striga* infestation at multiple locations in the country will follow before varietal release. Additionally, laboratory characterization of resistance mechanisms as those described in Chapters "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum", "Histological Analysis of *Striga* Infected Plants" and "*Striga* Germination Stimulant Analysis", and underlying genetic changes in these mutants as in Chapter "Identification of Closely Related Polymorphisms with *Striga* Resistance Using Next Generation Sequencing" will help determine the uniqueness and utility of these mutations. Alternative screening protocols for gained *Striga* resistance through physical mutagenesis in rice and sorghum in Burkina Faso are described Chapter "Screening for Resistance to *Striga Hermonthica* in Mutagenized Sorghum and Upland Rice in Burkina Faso" and for maize and rice in Madagascar in Chapter "Phenotyping for Resistance to *Striga Asiatica* in Rice and Maize Mutant Populations In Madagascar".

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Phenotyping for Resistance to *Striga Asiatica* in Rice and Maize Mutant Populations in Madagascar

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Abstract *Striga asiatica* is a major constraint to rain-fed rice (*Oryza sativa* L.) and maize (*Zea mays* L.) production in the Middle West and South regions of Madagascar. This work aims to establish a robust and efficient field and glasshouse screening protocol for identification of rice and maize mutants having resistance/tolerance to *Striga asiatica* by assessing the host plant phenotypic response under severe *S. asiatica* infestation in soil. Our screening strategy involved field and glasshouse experiments where soils were artificially infested with *S. asiatica* seeds under fertilizer-free growing conditions. Two maize (Plata and IRAT200), and two rice (B22 and F154), farmer-preferred *Striga* susceptible varieties, gamma irradiated at different doses were screened in a *Striga* endemic area of the Middle West and compared to their respective unmutagenized lines. Rice M3 to M5 progenies were also screened in *Striga* infested pots in a glasshouse. Developed protocols enabled the production of promising new *Striga* resistant/tolerant varieties in both maize and rice adapted to Malagasy agriculture.

Keywords *Striga asiatica* · Mutation breeding · Resistance · Maize · Upland rice · Madagascar

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Introduction

The large island nation of Madagascar has several agroecologies. The East is the wettest benefiting from trade winds that bring on annual average 4000 mm of rainfall. The North experiences seasonally heavy rainfall due to cyclones while the South and West are dependent on less frequent anti-cyclonic rains, with the Southwest receiving as little as 350 mm of annual rainfall and prone to devastating droughts (Rigden et al. 2022). *Striga asiatica* came to the island in the early 20th Century, likely with grain imports, and has now become a major cereal production constraint mainly in Middle West (Scott et al. 2020). Located between the Central Highlands and the West Coast at an elevation of 900–1300 m above sea level, this area of undulating plateaus called "*tanety*" is cut by lowlands and deeply eroded areas (Ripoche et al. 2019). The annual rainfall here is about 1100 mm, which is erratically distributed mainly during the hot season from November to April. The ferralitic soils of the *tanety* are low in organic matter and nutrients (especially N and P), acidic (average pH 5.3) and prone to erosion (Randrianjafizanaka et al. 2018). While the *tanety* have been used by subsistence farmers to grow upland rice and maize, traditional low input agricultural practices and *S. asiatica* often leaves them unfit for cereal production after a few years (Scott et al. 2021). Although conservation agricultural practices like no-till rice-maize rotations with perennial legume intercropping can, in the long run, lead to sustained cereal productivity in the *tanety* (Randrianjafizanaka et al. 2018), they are rarely permanently adopted by Malagasy farmers here (Razafimahatratra et al. 2021).

The most important staple cereal crop in Madagascar is by far rice, which is consumed daily with every meal in almost every Malagasy household. Rice accounts for approximately 46% of daily caloric intake of all Malagasies (Rigden et al. 2022), and more than half of that of rural people (Ozaki and Sakurai 2020). Following the stories of Burkina Faso (Chapter "Screening for Resistance to Striga Hermonthica in Mutagenized Sorghum and Upland Rice in Burkina Faso") and Sudan (Chapter "Mutation Breeding for Resistance to Striga Hermonthica in Sorghum and Rice for Sustainable Food Production in Sudan"), national rice production is insufficient to meet national need and therefore must be imported at an annual cost of $180 million in 2020 (TrendEconomy 2021). Rice is produced on the island mainly (> 90%) in wet irrigated coastal areas but upland rice, with government encouragement, is increasingly contributing to national production (Rakotoarisoa et al. 2019; Ozaki and Sakurai 2020). Unfortunately, this upland rice is vulnerable to *S. asiatica.* In uncontrolled conditions on the *tanety*, yield losses to *Striga* in upland rice average 73% (Rodenburg et al. 2020).

Maize is of lesser national importance than rice, but still the second most cultivated cereal in Madagascar with 225,000 t grown on 127,000 ha in 2020 (FAOSTAT 2022). It was initially used mainly as animal feed, but now accounts for nearly 5% of daily caloric intake (Rigden et al. 2022). Maize has become the third staple after rice and cassava and is particularly consumed in gruels by Malagasy children (Razafindratovo and Raveloarimalala 2021). Maize production has expanded an average of 2%

annually under a "National Maize Project" launched by the Malagasy government in 2004, though it is currently down from its peak in 2012 (Palchetti et al. 2021). Main production areas for Malagasy maize include the Middle West *tanety* where it is often rotated with upland rice and its yield loss to *S. asiatica*, where uncontrolled, averages 80% (Rodenburg et al. 2020).

Both rice and maize production in the erosion and *Striga*-prone *tanety* are demonstratively improved with conservation agricultural practices (Randrianjafizanaka et al. 2018; Rodenburg et al. 2020). These gains were even more pronounced when *Striga* resistant rice (Randrianjafizanaka et al. 2018; Scott et al. 2021) and maize (Andriamialiharisoa 2019) varieties are incorporated into the conservation agricultural practice. *Striga* resistance or tolerance is currently available in certain NERICA and in one farmer discovered rice variety called "Jean Louis" (Rakotoarisoa et al. 2019). Currently no *Striga* resistant maize varieties are adapted to the Malagasy Middle West where *S. asiatica* is a major concern to farmers (Autfray et al. 2022). We undertook this mutagenesis project in collaboration with the IAEA in the hopes of introducing *Striga* resistance into popular rice and maize varieties adapted to the *tanety*.

The Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Department of Nuclear Sciences and Applications, Plant Breeding and Genetics programme has supported the country's national crop improvement program helping to apply plant mutation breeding techniques to develop new cereal mutant lines since 1998. A Coordinated Research Programme CRP under close collaboration with the International Atomic Energy Agency (IAEA) was established in 2016 to support the *Striga* management in the country in order to develop new rice and maize mutant lines with enhanced *Striga* resistance. To realize gains from mutation breeding to cereal productivity in Madagascar, it is important to develop efficient methods for screening maize and rice after mutagenesis for both continued adaptation to local culture and select for any gained *Striga* resistance. The main objective of this document is to present established robust and efficient field and glasshouse screening protocols for identification of rice and maize mutants with resistance/tolerance to *Striga asiatica* under artificial *Striga* infestation.

Protocols

Collecting Striga asiatica *seeds.* In order to conduct field and pot experiments using artificial infestation sufficient to differentiate resistant maize and rice plants from susceptible ones, sufficient quantities of good quality *S. asiatica* seeds are needed. They must therefore be collected at the proper time and place and stored in a way that preserves their germinability. The life cycle of *S. asiatica* is similar to *S. hermonthica*, but the plants are smaller and flowers are scarlet rather than purple (Fig. 1). Seeds are best harvested from an area near where the screening takes place. Capsules (fruits) mature as the flowers die and fall off. Seed is mature when the capsules turn brown. Bundles of stems should be collected in tight plastic mesh bags just after most

Fig. 1 *Striga asiatica* plant development: **a** vegetative stage, **b** flowering stage, **c** post-flowering stage with green capsules, **d** mature stage where capsules are dried and seed is fully mature

capsules have turned brown (Fig. 2). In Middle West Madagascar where upland rice and maize are grown, this period generally occurs in late June. The bags are ideally hung in an open shelter with a roof and plenty of air circulation so that they can quickly dry. They should be kept in these bags, protected from excessive humidity and temperature until they are fully dry. Like *S. hermonthica*, seeds of *S. asiatica* have a dormancy period such that they will not germinate in the season in which they were produced. Therefore, *Striga* seed should be collected at least one year previous to their intended use.

Once the harvested *Striga* stems are fully dry, they may be removed from the mesh bag and threshed over a pan by rolling them between the palms of your hands or by picking the capsules from the dried stems (Fig. 3). Any seed left in the bag after removing the stems can be added to the collection pan. The capsules can be gently crushed with a mortar and pestle to dislodge any remaining seeds. The material in the pan is then passed through a series of sieves to separate the seeds from the capsules and other debris. Sieve size should begin at 2 mm, followed by 300 μm then 250 μm and finally 180 μm until only *Striga* seed remains. The seed can then be transferred to a storage container such as the magenta boxes shown in Fig. 3 which are then put into a second container (large enough to fit the container of seed) into the bottom of which activated silica gel desiccant is placed to keep the seed dry during storage for up to several years at room temperature.

Conditioning and germination test for Striga asiatica *seeds.* Prior to all experiments, the *Striga* seed batch germination rate should be checked. As with *S. hermonthica*, *S. asiatica* seeds that are at least one year old (after-ripened) need to be surface sterilized under aseptic conditions and allowed to condition for two weeks before testing their germination in the laboratory. Under a laminar flow hood, line the bottoms of three sterile 100 mm petri dish each with two sterile 90 mm Whatman No. 1 filter paper circles. Transfer an aliquot of 50 mg dried *S. asiatica* seed to a 2.5 mL Eppendorf tube and add 1 mL of 70% ethanol. Cap the tube and shake for 1 min. Under a laminar flow hood, remove the alcohol and add 20% (v/v) of household bleach (5.25% NaOCl) in water. Keep the seeds in this solution for 15 min, shaking every 5 min. Rinse the seeds five times with sterile distilled water (ddH_2O). Distribute the surface sterilized *Striga* seeds across the surface of the filter papers in the three petri dishes. Add 5 mL of ddH_2O to each to soak the filter papers. Seal

Fig. 2 Mature *Striga asiatica* plants collected and stored in a tight plastic mesh bag

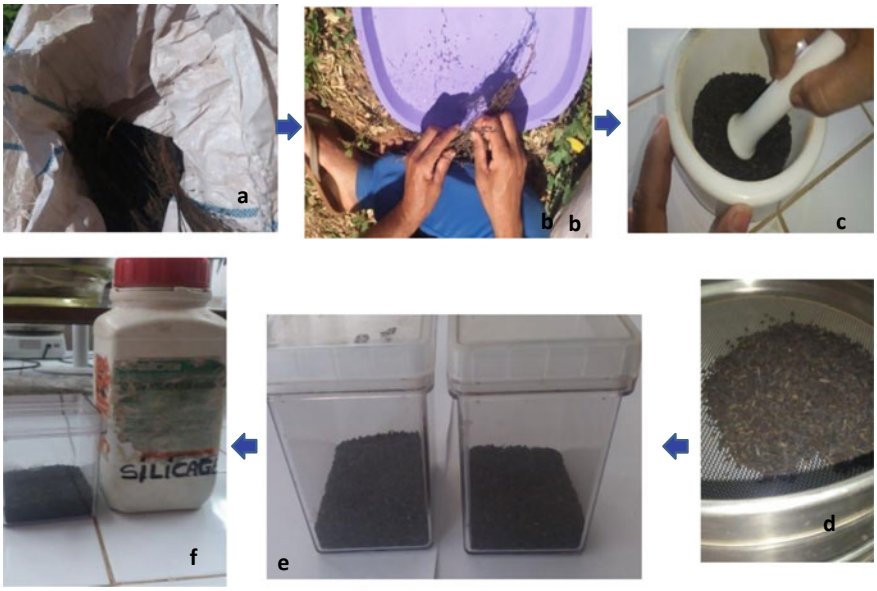

Fig. 3 Process of threshing and cleaning *Striga asiatica* seeds: **a** Dried *Striga* stems with capsules are removed from the tight plastic mesh bag in which they were collected, **b** seeds are gently threshed by hand over a shallow pan, **c** capsules are gently ground in a mortar and pestle to dislodge seed, **d** threshed material is sieved until only seed remains, **e** seeds are transferred to storage containers **f** the storage containers are placed in a desiccator over silica gel

the plates with parafilm and incubate at 30 °C for 14 days in a dark incubator to condition the *Striga* seed.

After the seeds have conditioned for two weeks, open the plates under a laminar flow hood and add 5 mL of 1 ppm GR24, a synthetic strigolactone that stimulates *Striga* germination. Seal the plates with parafilm. Incubate for 1–2 days at 30 °C in darkness. Count germinated *Striga* seeds under a dissecting microscope. Divide the number germinated by the total number of seeds in a viewing field in multiple fields across the three plates to determine germination rate of that batch of *S. asiatica* seed.

Preparing an inoculum for pot and field screening. If the *Striga* seed germination rate determined from the laboratory check of the batch is at least 50%, the batch is suitable for screening purposes in field or greenhouse. Before using it for such, mix it with an equal volume of fine dry sand and return to the desiccator until use (Fig. 4).

Plant material used and irradiation treatment. The upland rice varieties chosen for mutagenesis were B22, a line originally from Brazil but widely grown in Madagascar, and F154, a variety developed by FOFIFA, the Malagasy national agricultural research entity. The maize used were Plata and IRAT200, both yellow OPVs (Fig. 5). All chosen varieties are farmer-preferred (yield, growth habit, grain quality), but sensitive to *S. asiatica* and adapted to the Middle West *tanety* where *Striga* is particularly problematic.

Fig. 4 *Striga asiatica* seed inoculum, 50:50 mix of cleaned *Striga* seeds with fine dry sand

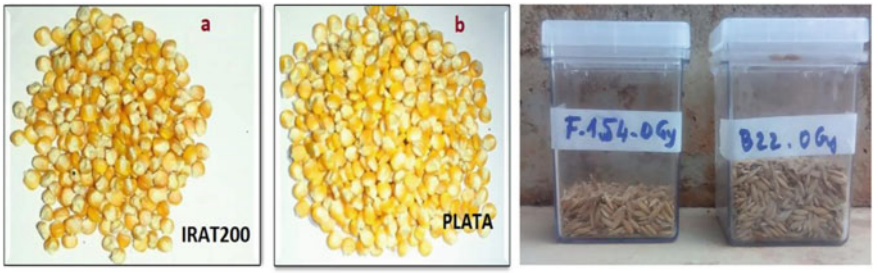

Fig. 5 Varieties mutagenized by gamma irradiation and derived populations screened in Madagascar. From left to right, yellow maize IRAT200 and PLATA and upland rice F154 and B22

In order to increase genetic variability within these rice and maize genotypes, 1000 seeds of each were gamma irradiated at various doses at the Plant Breeding and Genetics Laboratory of the Joint Food and Agricultural Organization of the United Nations International Atomic Energy Agency (FAO/IAEA) Division of Nuclear Techniques in Food and Agriculture at Seibersdorf, Austria using a ^{60}Cobalt source.

(van Harten 1998; FAO/IAEA 2018). Radiosensitivity tests of each variety was done prior to all experiments to determine the appropriate dosages (Chapter "Physical Mutagenesis in Cereal Crops"; van Harten 1998; Razafinirina 2011; Rakotoarisoa et al. 2017; FAO/IAEA 2018). Effective mutagenic but non-lethal doses chosen were 100, 200, 300 Gy for maize Plata and B22 rice varieties, and 100 and 200 Gy for maize IRAT200 and F154 rice varieties. Mutant populations derived from these mutagenized materials were screened and advanced in Madagascar first in the field and subsequently in greenhouse pots.

Field screening. Screening of early generations after mutagenesis (M2/M3) was done at the Station of Kianjasoa, Regional Research Center of the Middle West FOFIFA. The station is located 190 km from Antananarivo, the capital of Madagascar, in the district of Tsiroanomandidy of the Bongolava region. Coordinates are: 49°22′62.1″ East longitude, 19°03′20.3″ South latitude at 899 m above sea level. Both maize and upland rice production are constrained by *S. asiatica* in this region due to erratic rainfall and poor soils prone to erosion. Organic matter (1.7%), phosphorous (< 2.5 ppm) and nitrogen 0.25%) in the plots were all suboptimal. Screening was performed without nutrient inputs, typical of traditional farming in the region that favors *Striga* infestation (Andriamialiharisoa 2019; Rakotoarisoa et al. 2019). Soil was ploughed in advance by a tractor and hand weeded where necessary just before plots were marked and planting holes dug by hand hoe (Fig. 6).

The experiments were planted at the corresponding regular seasons for maize and rice in the region into 8 cm deep holes spaced 25 cm apart for rice and 70 cm for maize (see details below) into which 0.05 g of the sand/*Striga* inoculum (50:50, v/v,

Fig. 6 Field preparation, **a** ploughing the land, **b** hand-weeding, **c** setting plots, **d** digging planting holes

approximately 5000 weed seeds) was added to ensure uniform and heavy infestation. This was covered by 3 cm of soil before sowing the cereal. Each crop seed was covered by 3 cm of additional soil (Fig. 7). Five meter row plots were used for sowing M2/M3 rice seeds and 10 m for the maize seeds per variety, per initial irradiation dose, replicated in three plots for each rice entry and four plots per tested maize variety (Fig. 7). Plots were labeled with the name of the variety and the dose of irradiation. The same plot layout in the same area of the field was used in the subsequent (M3) generation screening with additional infestation to ensure high *Striga* pressure.

Harvest and maintenance of M2/M3 seeds. Assuming that the first generation screened in the field was at the M2 generation, certain individuals may be selected based on apparent improved performance (e.g., fewer emerged *Striga*, less host plant damage) over their unmutagenized counterparts (0 Gy). These selected individuals need to be advanced to the M3 for further testing. This requires self-pollination. For rice, no special techniques are needed since self-pollination occurs within each spikelet before anthers emerge. However, to ensure self-pollination, a small bag may be put over the panicle before anthers emerge. This bag should be left on until harvest. In maize, which is a naturally cross-pollinated species, self-pollination requires some effort. As the uppermost ear begins to emerge, usually when tassels have fully emerged and expanded, but before anthers emerge, cover the ear shoot with

Fig. 7 Field screening of maize and rice for *S. asiatica* resistance: **a** artificial infesting planting holes with *Striga* inoculum, **b** sowing of cereal seeds, **c** labeling of planted plots (photo taken at maturity in maize plots), **d** labeling of the holes sown with rice or maize seeds of M2 or M3 families in the same row

a coin envelope or shoot bag before silks emerge. Leave the shoot covered for several days until silks emerge (usually when the first anthers are visible on the tassel). When dew dries from leaves in the morning, use a larger envelope or pollinating bag to collect pollen (gently bend into the pollinating bag with opening facing up and shake tassel inside the bag to release pollen). Briefly uncover shoot on the same plant and immediately invert bag of collected pollen over the silks. Cover the ear with the pollinating bag and secure by folding bottom edges around the stalk and stapling. Leave bag until harvest.

When selected plants are mature, cut rice panicles or maize ears and dry in paper envelopes within mesh bags labeled with plot number, plant number, variety, original radiation dose. Thresh or shell when harvested panicles or ears are fully dry and store seeds in labeled envelopes in plastic bags in a refrigerator (4–8 °C) until use.

Details of the screening plot plantings. Sow up to 20 M3 rice seeds from each selected M2 plant into a single 5 m row following the field layout and inoculation method described above with each planting hole spaced 25 cm apart. Plant one seed per hole. Leave 25 cm between rows such that spacing is 25 × 25 cm between plants. It is convenient to organize the M3 family rows by variety and radiation dose. Do the same for maize, but use 10 m long rows, with holes spacing of 70 cm within row and 70 cm between rows planting 14 M3 seeds from each selected M2. Repeat the same plantings in three replicate plots for rice and four for maize of each M3 family that was advanced from the selected M2 plants. You should therefore have up to 60 M3 plants from each selected M2 mutant and 56 M3 plants from each maize mutant to evaluate. Plant the corresponding control (parent variety-0 Gy) in two rows in the middle of each replicate plot as a check for phenotype comparison. After sowing, label each family row with the variety and original radiation dose as well as the number of the M3 family (Fig. 7).

Aside from clearing weeds before sowing (Fig. 6), hand weeding should be practiced during the crop vegetative stage at 30, 45 then 60 days after sowing (DAS), taking care not to damage *Striga* plants in latter days. A hoe may be shallowly used at the 30-day weeding since *Striga* is unlikely to emerge by then (Fig. 8). During the crop reproductive phase, weeds on plots should no longer be removed. *Striga* emergence counts and host sensitivity parameters (see below) are taken at flowering for both rice and maize. Selection of potential *Striga* resistant/tolerant mutant lineages to advance to further trials is based on comparisons with the unmutagenized (0 Gy) original parent line within each plot. Those lineages with apparently fewer *Striga* and/or fewer symptoms of *Striga* infection (less leaf firing, larger ears and grain) than the unmutagenized check are tested in subsequent field or pot trials.

Screening advanced rice mutant lineages in pots. Once the number of selected *Striga* resistant mutants from field screening is reduced to a manageable number, further screening and verification of resistance/tolerance can proceed in greenhouse pots. This way, more detailed observations are possible including comparisons to the original unmutagenized line with limited impact of environmental influences encountered in field trials. We used a well-vented greenhouse during the normal cropping season to evaluate advanced mutant rice lineages (M4–M6) in both rice varieties (B22 and F154). Temperature remained in the range of 28–38 °C in the

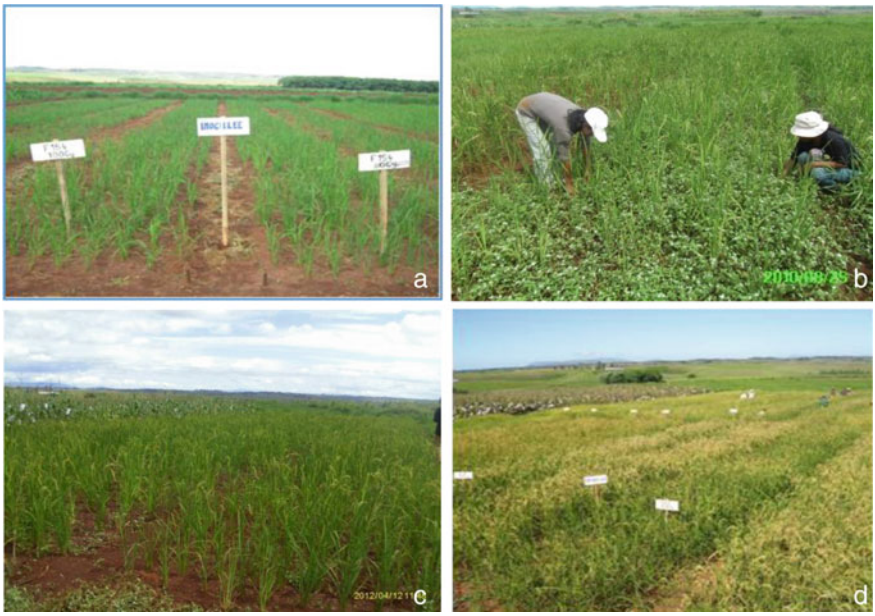

Fig. 8 Rice plots at **a** vegetative phase, **b** during hand weeding, **c** after weeding, **d** seed set

greenhouse during the pot trials. We did not find pot screening useful for maize as plants did not grow well in the greenhouse. Screening rice for *S. asiatica* resistance can be performed as follows.

In a 10 L plastic pot with drainage holes, put a thin sail (filter) in the bottom of each pot to prevent *Striga* seeds from coming out through the bottom holes (Fig. 9). Fill pots 2/3 to the top with local soil mixed with equal parts thin sand. Artificially infest pots by spreading 0.5 g of the *S. asiatica*/sand (50:50) inoculum described earlier in this chapter on the soil placed in the pot. This should put about 5000 germinable *Striga* seeds in each pot. Cover this *Striga* layer with enough of the same sand/soil mixture used in the bottom to raise the surface 4 cm. Add another *Striga*/sand layer as before and then add another 4 cm of the sand/soil mixture. Plant two rice seeds per pot and cover with an additional 3–4 cm of the sand/soil mixture and water until saturated. Continue watering to saturation two times a week for two weeks to ensure the rice seeds germinate and *Striga* seeds are conditioned. Thereafter, water once weekly until rice plants flower then stop watering to encourage *Striga* infection. Do not add fertilizer to pots, again to encourage *Striga* infection. This harsh selective pressure will help distinguish resistant from susceptible plants. Pull any weeds in pots besides *Striga* at 15, 30 and 45 days after sowing rice. Count the number of emerged *Striga* in each pot at these same intervals as well symptoms of infected rice plants (see below). Compare these measures on selected mutant lineages with those of infested and uninfested pots of unmutagenized counterparts. Arrange pots in a complete randomized design. Pots and soil may be reused in subsequent trials.

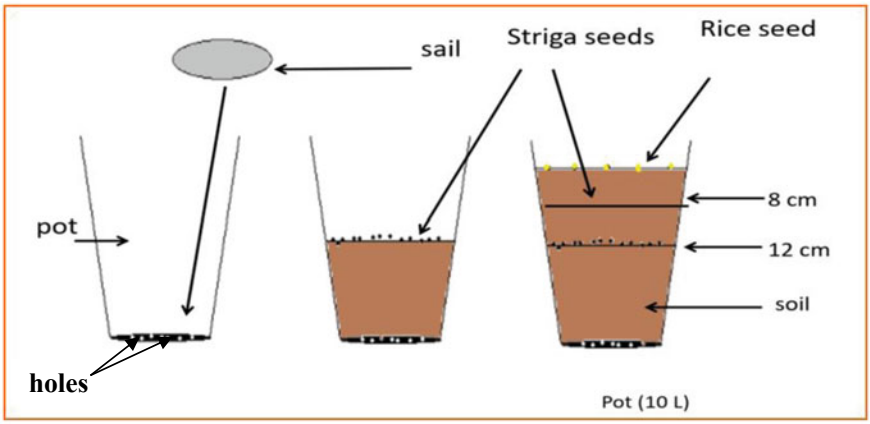

Fig. 9 Pot preparation for glasshouse screening of mutant rice lineages for resistance to *Striga asiatica*

General guidelines for screening maize and rice mutant lineages for S. asiatica *resistance and tolerance.* A general assumption in this mutagenesis breeding project is that gained *S. asiatica* resistance will be expressed by fewer emerged *Striga* around mutant maize and rice plants relative to their unmutagenized counterparts (0 Gy). Also, because there is less *Striga* infection around such plants, symptoms in resistant mutants will be less severe than those of *Striga* infected controls. All of the original rice and maize varieties chosen for mutagenesis are particularly sensitive to *Striga* infection which causes leaf chlorosis and firing (senescence), reduced shoot growth and fertility through reduced pollen production (smaller tassels in maize, smaller panicles in rice with fewer anthers) and reduced grain yield (smaller rice panicles and smaller or deformed maize ears). Since such symptoms, particularly reduced fecundity, can result from gamma irradiation itself, it is important to distinguish heritably reduced sensitivity to *Striga* from general reduced fitness due to mutation in genes controlling normal growth and reproduction. Mutants of the latter type are generally eliminated in early generation screenings. The goal of mutagenesis must be to improve *Striga* resistance and/or tolerance at no cost to overall plant productivity or adaptation to their target environment. Therefore, mutant lineages selected for advancement must show improved performance under *Striga* pressure relative to the original unmutagenized line from which they were derived with equal or better performance to the original line in uninfested conditions. It is therefore important to compare selected mutants with potential gained *Striga* resistance in both *Striga* infested and non-infested conditions at some point during the screening process in their target environment.

Because all the original rice (B22 and F154) and maize (IRAT200 and Plata) varieties are particularly sensitive and susceptible to *S. asiatica*, with multiple parasites eventually emerging around host plants with fairly severe symptoms (prematurely dying leaves and low yield) under *Striga* infestation, selection for *Striga* resistance/

tolerance began in early generations (M2 and M3). Verification was then done on advanced progeny in the selected lineages (M4 and M5). Under the severe selection pressure described earlier for field screening (*Striga* inoculum added to each planting hole and no fertilizer applied to plots), seeds were only harvested from plants of mutant lineages having fewer emerged *Striga* and less affected leaf area and more grain than unmutagenized counterparts for advancement to subsequent trials (field or pot) to verify their gained resistance and tolerance. In these verification trials, specific phenotypic characters were observed and recorded including the *S. asiatica* plants number emerging above the soil per plant, counted at host flowering, the survival rate per genotype (number of surviving plants divided by the total number of germinated seeds multiplied by 100), the maximum plant height, the number of rice tillers and fertile panicles or ear numbers produced per maize plant, the 100 (for maize) or 1000 (for rice) seed weights, the number of leaves showing premature senescence expressed as a percentage of total number of leaves per plant and for maize, the ear shape. Figures 10 and 11 show examples of observed phenotypes in maize trials and Fig. 12 shows rice examples.

From these detailed observations, advanced mutant lineages can be classified according to their performance under *Striga* infestation. We used modifications of those classes described by others (Kim 1991; Sinebo and Drennan 2001) without clear distinction between resistance and tolerance characters since the latter under

Fig. 10 Examples of maize performance under *S. asiatica* infestation. **a** Susceptible maize plant with most leaves affected, **b** a highly susceptible maize plant with premature shoot death, **c** a highly resistant maize

Fig. 11 Various maize ear types observed under *S. asiatica* infestation to classify resistance. **a** Ears from a moderately resistant mutant having small ears with empty rows and kernels arranged in disorder, **b** ear from a resistant mutant with < ¼ affected with slight blackening and curving at tip, **c** ear from a highly resistant mutant, large with full, well-arranged kernels. **d** Multiple ears from an advanced resistant mutant line, IRAT200Gy-L9-2-1-X22, harvested from uninfested plots in yield trials after selection

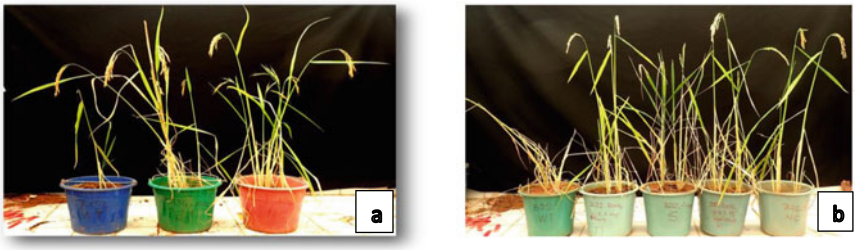

Fig. 12 Examples of rice performance in greenhouse pots. **a** original F154 rice line (left) in *Striga* infested pot, resistant F154 mutant (center) in *Striga* infested pot and original F154 rice line (right) in uninfested pot. **b** From left to right, original B22 rice line, a moderately resistant B22 mutant, a susceptible B22 mutant, a resistant B22 mutant in pots infested with *Striga asiatica.* The fifth pot contains the original B22 without *Striga.* All mutant lineages are at the M6

the high infestation conditions employed was largely a function of the former. Table 1 gives a summary of this classification system.

Resistance among the selected mutant lineages in this mutation breeding exercise may involve genes contributing to both tolerance and resistance to *S. asiatica.* Distinguishing alleles resulting specifically in tolerance would require detailed comparisons of the same mutants grown with and without *Striga* (see Chapter "Screening for Resistance to Striga Hermonthica in Mutagenized Sorghum and Upland Rice in Burkina Faso"). The end result, however, was that selected maize (Table 2) and rice (Table 3) mutants show improved yield under *Striga* infestations over their original varieties. Generally, these mutants appear to be resistant to *S. asiatica*, in that they supported fewer parasites than their unmutagenized counterparts. Several of the M5 selections are being propagated for evaluation in replicated field trials for possible new variety release in Madagascar.

Conclusion

The protocols described in this chapter were successfully used to select *Striga* resistant mutants from gamma irradiated maize and upland rice varieties adapted to the *tanety* of Middle West Madagascar where *S. asiatica* is a serious production constraint. Introducing *Striga* resistance through mutagenesis in cultivars adapted to these conditions can help to sustain maize and expand rice production in the country, especially if used in combination with conservation agricultural practices that reduce erosion and improve soil fertility in this fragile agro-ecology. Mutation breeding with selection based on the protocols described has resulted in 19 maize varieties in two farmer-preferred backgrounds that are superior to the original varieties in terms of resistance and/or tolerance to the local strain of *S. asiatica.* Similarly, 34 *Striga* resistant/tolerant upland rice varieties in two popular genetic backgrounds were identified and advanced through a combination of field and greenhouse screenings among the progeny of mutagenized seed. Further characterization in both laboratory assays (Chapters "An Agar-Based Method for Determining Mechanisms of Striga Resistance in Sorghum"–"Striga Germination Stimulant Analysis") and multi-location field trials of the selected *S. asiatica* resistant maize and rice varieties continues with much promise for improved cereal production in Madagascar. Similar efforts are described in Chapter "Screening for Resistance to Striga Hermonthica in Mutagenized Sorghum and Upland Rice in Burkina Faso" for sorghum and rice in Burkina Faso and in Chapter "Mutation Breeding for Resistance to Striga Hermonthica in Sorghum and Rice for Sustainable Food Production in Sudan" for sorghum and rice in Sudan.

Table 1 Adapted method for assessing the resistance of tested maize and rice genotypes under severe *Striga asiatica* infestation

Plant[a] reaction to *Striga*	Score	No. emerged *Striga* / plant	(%) Affected leaves / plant	(%) infected ears or panicles / genotype	Ear or panicle symptomatic description	Symptoms at harvest	Plant growth
HR	0	0	0–10	0–10	Healthy, normal-sized maize ear or rice panicle	Full maize ears or rice panicles	Normal plant height and growth rate
R	1	0–2	11–20	11–20	< ¼ of the maize ear or rice panicle is affected but generally with normal size	Little curvature with slight blackening and reduced kernels at the upper quarter of the maize cob, rice panicles mostly complete	Few visible leaf symptoms, near normal height and growth
MR	2	2–4	21–40	21–40	¼–½ of the maize ear is affected and generally small ear and tassel size, rice panicles may show some sterility	Curvature, blackening and reduced kernels at the upper half of the maize cob with some empty rows and disordered arrangement, rice panicles with slightly reduced grain numbers	Slight but noticeable stunting

(continued)

Table 1 (continued)

Plant[a] reaction to *Striga*	Score	No. emerged *Striga* / plant	(%) Affected leaves / plant	(%) infected ears or panicles / genotype	Ear or panicle symptomatic description	Symptoms at harvest	Plant growth
MS	3	4–6	41–60	41–60	Up to 3/4 of the maize ear affected with size reduction, noticable sterility of rice panicle	Curvature of the maize cob with blackening and incomplete or empty rows arranged in disorder, reduced ear and tassel size, rice panicle fertility rate slightly higher than sterility	Moderate stunting
S	4	6–8	61–80	61–80	Maize tassel small with reduced pollen, ears very small or number reduced. Rice panicle shows high sterility, fewer tillers	Maize ears very stunted and often slightly curved often with blackening and incomplete, disordered and/or empty rows, rice panicles reduced or nearly empty	Plants short and growth retarded
HS	5	> 8	> 80	> 80	Little or no pollen production	No useful maize ears or rice panicles formed	Stunted, or growth stops with premature death

[a] *h*—highly resistant, *R*—resistant, *S*—susceptible and *HS*—highly susceptible

Table 2 Total number of maize plants selected and advanced for further testing at each generation after mutagenesis

Variety-original radiation dose	No. selected plants at **M2**	No. selected plants at **M3**	No. selected plants at **M4**	No. selected plants at **M5**
Irat200–100 Gy	630	250	65	18
Irat200–200 Gy	670	204	70	22
Plata–100 Gy	640	260	68	40
Plata–200 Gy	587	245	85	42
Plata–300 Gy	610	238	115	45

Table 3 Total number of rice plants selected and advanced for further testing at each generation after mutagenesis

Variety-original radiation dose	No. selected plants at **M2**	No. selected plants at **M3**	No. selected plants at **M4**	No. selected plants at **M5**
F154–100 Gy	500	180	15	8
F154–200 Gy	500	171	8	5
B22–100 Gy	500	195	15	9
B22–200 Gy	500	188	14	8
B22–300 Gy	500	145	20	5

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An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum

Patrick J. Rich

Abstract This chapter describes an agar-based method for co-culturing sorghum and *Striga* that can be used to determine *Striga* germination stimulant activity, haustorial initiation activity and other pre-attachment and early post-attachment resistance mechanisms in sorghum.

Keywords *Striga* · Sorghum · Bioassay · Mechanisms of resistance · GR24

Introduction

Having evidence of gained *Striga* resistance in mutagenized cereals through field and pot studies, it is useful to determine what mechanism underlies the resistance reaction. This is particularly important in the context of sustainability and integrated *Striga* management, whereby the crop resistance is combined with other control methods, e.g., soil nutrient enhancement through fertilizers, water conservation practices, post-emergent weed control practices or with other resistance traits (Rich 2020). Extended reliance on a singular host plant resistance trait may lead to selection of virulent *Striga* populations that are able in a few generations to overcome the acquired resistance from mutagenized crop varieties. Determining the underlying mechanism leading to the reduced number of parasites emerging around field and pot grown plants is most easily done in a medium in which the *Striga*-host interactions can be observed at their earliest stages with repeated, non-destructive observations.

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Protocols

This section tells how to prepare plates of *Striga* seed embedded in 0.7% agar beginning with surface sterilization and conditioning of the *Striga* seed. It builds on an agar gel assay for determining *Striga* germination stimulant activity published decades ago by Hess et al. (1992). In this assay, since one is observing a limited number of *Striga* seeds, it is important to choose a weed seed source that germinates fairly well, at least 30% in 10^{-8} M GR24. Ideally, the source of *Striga* seed should come from the target environment in which the resistant cultivar is to be grown. The following procedures are intended for determining sorghum reactions to *Striga asiatica* or *S. hermonthica*. They may be adaptable to maize or rice but modifications to suit screening of those crops is not covered in this chapter.

Conditioning Striga *seed*. Prepare a Benomyl (a benzimidazole fungicide, Sigma-Aldrich) 100× stock solution by dissolving 0.15 g Benomyl wettable powder in 10 mL DMSO. Heat (50–70 °C) to completely dissolve the fungicide in the solvent. Store at room temperature in a brown glass bottle for up to three months.

Prepare Metricide 28® solution (2.5% glutaraldehyde; Metrex Research Corp.). A small volume can be activated by adding 1.2 mL of activator to 100 mL of the bulk Metricide if only a few batches of *Striga* will be conditioned over the period. In a biosafety cabinet or on a lab bench, but outside the (laminar flow) transfer hood, remove an aliquot of cleaned (dry but sieved to remove plant material other than seed) *Striga* seed from bulk to a 50 mL flask. This should be done on a heavy white blotter soaked with bleach to contain any dry seed which might miss the flask during the transfer. The normal amount for this size flask is usually six smidgen scoops, enough for about 100 plates. You should be able to get about 15–18 100 mm plates for each smidgen of clean weed seed. Less can be used, but if you use more than six scoops, you will need to perform the surface sterilization steps in a 125 mL flask, doubling the volumes of sterilants (ethanol and Metricide), washes and conditioning solution. Add 25 mL 75% ethanol to the seed in the flask. Wash the outside of flask with bleach. Move the flask with seeds in alcohol to a sterile (laminar flow) transfer hood (also onto a light colored blotter soaked with bleach) and with a sterile glass pipette equipped with an amber bulb, agitate for 2 min by sucking the solution and weed seed up and down in the pipette repeatedly. Let the seed settle in the flask. If sand or heavy debris is visible at the bottom of flask, use the pipette to suck that up. You will also unavoidably suck seed into the pipette with the sand but once the sand settles in the pipette, the sand will be at the bottom and you can remove it by squeezing over the waste flask filter funnel until you reach the seed layer. Add this back to the flask of seed in the 75% ethanol. Let the seed settle again and then slowly pour the liquid off into the waste flask filtered funnel until mostly seed remains. Use the pipette to suck off the remaining liquid by squeezing the bulb before putting into the seed slurry and pressing the tip against the inside bottom of the flask, then slowly releasing the bulb. Properly done, this will suck up mostly liquid and very little seed.

Add 25 mL of activated Metricide. As before, agitate for 2 min by sucking seeds and sterilant in and out of the pipette under the surface of the liquid. Remove the

bubbles from the surface of the Metricide solution with the pipette before emptying the liquid to the waste flask in the same manner as the alcohol was removed, pouring off most, then sucking remainder off with the pipette. Rinse 2× with sterile distilled deionized water (ddH_2O), each time agitating with the pipette for about a minute then removing the liquid as before.

Add 25 mL ddH_2O and 25 μL of the 100× Benomyl stock to flask. This is the conditioning solution. Cover the mouth of the flask with Aluminium foil and put in darkness in an incubator set at 29 °C. **Change Benomyl solution after 1 day** and then every 2–3 days after until the seed is embedded in agar.

Pouring the plates. You may embed the *Striga* seed in agar after 5 days in the conditioning solution and allow the conditioning to finish in the agar. It is usually most convenient to do this a day or two before planting the sorghum. **Do not use the seed until it has been in a combination of solution and agar for a total of 10 days**. Germinability is maximal from 10 to 15 days after the conditioning start date.

Prepare agar (0.7%) to embed *Striga* seeds (six smidgen scoops of seed should yield about 100 plates). Prepare bottles of agar in 1 L Pyrex bottles, each containing 900 mL water + 6.3 g BactoAgar. Each bottle should be enough to pour twenty-five 15 × 100 mm plates (35 mL/plate) or fifteen 25 × 100 mm plates (60 mL/plate). Autoclave the bottles of agar on a liquids cycle with 15–20 min sterilization time, then allow to cool in a water bath to 50 °C (turn on water bath when autoclave cycle starts).

To distribute the seed properly and uniformly on each plate takes some practice. Ideally you want around 20 *Striga* seeds per square cm (more if germinability is low). Use a glass Pasteur pipette, the same kind used during the initial surface sterilization and changing of Benomyl. After changing the Benomyl solution, suck as much seed into the pipette from the conditioning flask as possible with the aid of an amber bulb and let it just settle to the narrow tip. Under sterile transfer hood, set out 20 plates (or whatever amount conveniently fits in the front of your hood) and open them. Add a drop of settled *Striga* seed from your pipette by touching it to the center of the opened plate bottom and barely squeeze the bulb to deliver an amount of wet seed about the size of a sorghum seed to each plate, trying to be consistent between plates. The amount of *Striga* is the same whether you are using 15 or 25 mm Petri dishes.

After distributing seed to all plates in a set, add more of the conditioning solution from the flask of conditioned seed to dilute the drop of seed. Compare the density of seed in each drop and add or remove seed from plates until all look to have a similar amount of seed (Figs. 1 and 2). Be sure not to let any seed dry out during this distribution process. After seed has been put in all the plates in roughly equal portions, remove the bottle of sterile agar cooled in a water bath to 50 °C, spray the outside with 75% ethanol and then bring it under the hood and open it. In a smooth and steady manner, pour the liquid agar directly over the *Striga* seed keeping the mouth of the bottle as close to the plate as possible and not interrupting the flow until just after the agar reaches the sides of the plate. The volume of agar should be about 35–40 mL for 15 × 100 mm plates or 60 mL for 25 × 100 mm plates (the level of the agar in the plate should appear to be about 2/3–3/4 full, Fig. 3) and the *Striga* seeds distributed evenly across the plate (Fig. 4). Using the deeper plates will allow

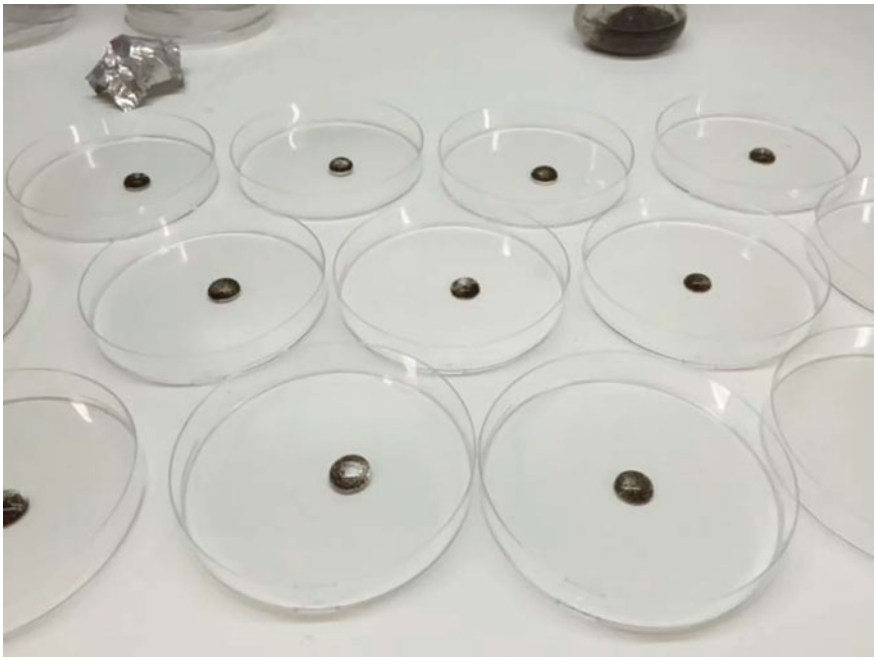

Fig. 1 Try to distribute *Striga* seed equally among plates. Keep the seeds always covered with liquid

the sorghum shoot more space to grow if you plan on keeping them long enough for early post-attachment observations (21 days or longer after plating sorghum). Allow plates to cool 10–15 min before covering. Continue this process until all the plates you need for that set are poured.

Record the conditioning start date and the date the seed was embedded in agar for each batch of poured plates. Stack 18–20 plates, making sure lids of each are securely fitted. They can be left in the laminar flow hood so long as the blower is turned off. The blower will dry them out. They can also be left on a lab bench if they will be used within a day or two. If they were poured early and won't be used for a few days, they should be closed inside a plastic bag, most conveniently the sleeve in which the new plates were originally packaged, to maintain high humidity during the remaining conditioning period and placed in a dark 29 °C incubator until they are ready to use (between 10 and 14 days from the conditioning start date of the *Striga* seed).

Surface sterilization of sorghum seed. Determine which accessions you will test according to how many *Striga*-in-agar plates you have prepared and what you can reasonably read at each observation period. Always include at least one check entry with known high *Striga* germination stimulant activity (most important), and another with low *Striga* germination stimulant activity. These will allow you to compare between sets of experimental entries (accessions) as you screen your collection. I

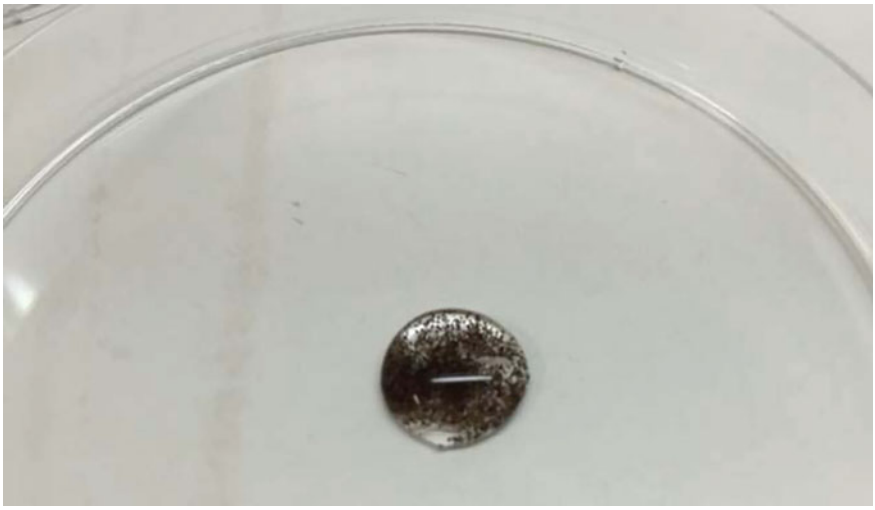

Fig. 2 In a drop of conditioning solution with a 1 cm diameter, the seed density should look similar to this

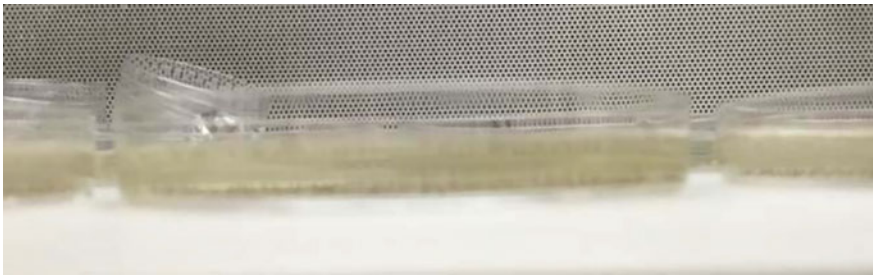

Fig. 3 The agar level should fill the bottom of the petri dish 2/3–3/4 full

usually try to keep the number of accessions to about 30 in a set so that with three plates per accession, I am not overwhelmed on observation days and I can fit all 90 or so plates on a large tray on a single shelf in our incubator.

Deglume sorghum seeds and remove any debris or immature, uncharacteristic or damaged kernels. Record seed source (year and field row) and assign each entry a simple number. Count out 10–20 seeds and place in a 6 dram glass vial, 15 mL Falcon® tube or similar glass or plastic container. Having them all together in a rack is a convenient way to handle multiple entries. Once all entries are placed in vials, add approximately 5 mL of a freshly prepared 50% bleach/0.2% Tween® 20 (a polysorbate-type nonionic surfactant, Sigma-Aldrich) solution from a squirt bottle (for 500 mL, combine 250 mL household bleach [5.25% NaOCl] with 250 mL ddH_2O and add to this 1 mL of Tween 20, use this within 1–2 days). Cap the vials. Shake them either individually or together in the rack.

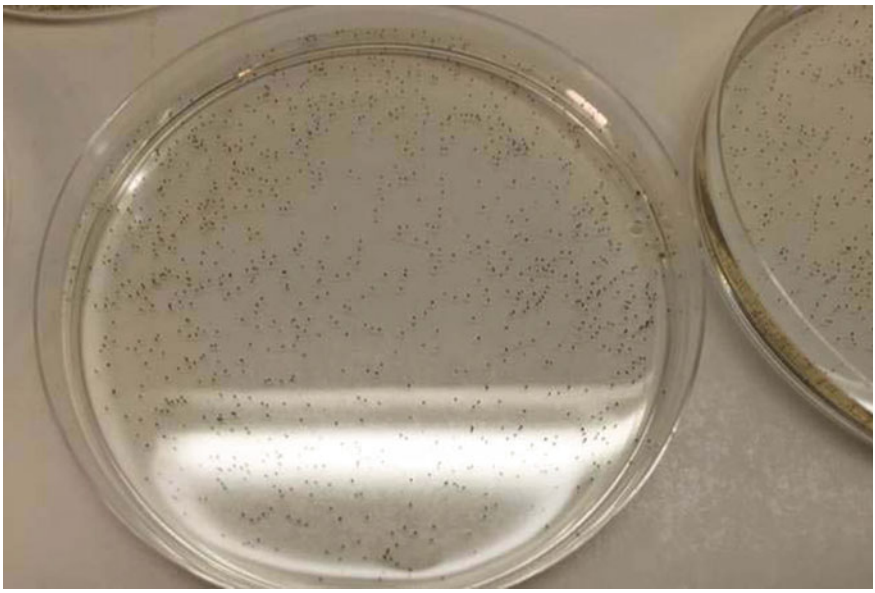

Fig. 4 If poured properly, seed will be evenly distributed in the agar at about this density. Ideally the seed density will average about 20 seeds/cm^2

Soak seeds in this solution for about 30 min (but don't leave more than 60 min). Shake them occasionally (two or three times) during this surface sterilization to assure uniform contact of bleach solution and break surface tension. Seeds which do not sink to the bottom may be bug eaten or damaged and will likely not germinate so they should be discarded. After the 30 min soak, move the vial(s) to a sterile transfer hood. Pour off the bleach solution into a waste container, a large beaker or better yet, a sidearm flask fitted with a funnel and lined with filter paper. Be careful not to pour out the seeds. If any are still floating, you will likely lose them but that's okay since any that float are probably not viable. After pouring off the bleach, fill the vial with sterile ddH_2O from another squirt bottle. Pour this off and repeat this water wash two more times to remove the bleach solution. Proceed with these washes for each vial of seeds. Leave the caps off.

Prepare a 5% w/v Captan 50 W (a phthalimide fungicide) solution by adding 25 g Captan powder to 500 mL sterile ddH_2O in a squirt bottle (use within 1 week). Wear gloves and a mask when weighing out Captan powder. The powder doesn't really dissolve in the water but rather forms a slurry. It is therefore important to shake the bottle often to keep the slurry at the appropriate 5%. Wear gloves when handling the Captan solution. Add approximately 5 mL of this Captan slurry to each vial, remembering to agitate the squirt bottle between vials. Recap the vials and let the seeds soak in this solution overnight to imbibe. They can be removed from the transfer hood as long as they are capped.

Germination of sorghum seed. Next day (or after at least 5 h of soaking in Captan) under a laminar flow hood, pour off Captan slurry (into the Captan waste container) after shaking the tube to resuspend the Captan. Take care not to dump the seeds. Add 5 mL sterile distilled water. Pour seeds and water into labelled sterile 100 mm petri dishes, each containing two sterile 90 mm germination paper circles (#SD3-3/8 from www.anchorpaper.com) in the bottom and another wetted one in the lid. Replace the plate cover (lined with germination paper). You may use autoclaved glass petri dishes so that they can be reused. If the seed are clumped together, spread them across the filter paper with gentle shaking or use forceps cleaned with alcohol to move them. Stack plates inside a plastic box with a loose fitting lid to retain high humidity but also allow air flow. Add wetted paper towels to the box to help maintain high humidity. Place this box in a warm dark place (30 °C incubator) overnight or until the seed has germinated and the radicle is around 1 cm long. Unfortunately, this varies among accessions so you may have to adjust the time in the incubator. One cm is ideal, but seedlings with roots as long as 2 cm and as short as 0.5 cm are still okay.

Plating sorghum into agar with embedded Striga. Next day, move plates containing germinated seeds to the laminar flow hood. Remove 100 mm plates containing *Striga* in agar (at least 8 days after conditioning start date, but not more than 18 days—optimal is 10–12 days). Remove atomizer containing 10^{-5} M GR24 (see inset "*How to prepare and keep a clean and active GR24 solution for applying to agar plates*") from refrigerator so that it can come to room temperature. Open each plate (under the hood) and wipe out any condensation that may have formed from inner lid surface. Label agar plates at the edge on both the top and bottom with the appropriate sorghum entry number. Use alcohol wiped forceps to puncture the agar and split it gently apart enough to accommodate the sorghum seed and radicle. Make this "planting hole" over the place where you marked the entry number. Gently pick up the germinated sorghum with the forceps, holding it by the kernel so as to not damage the emerging shoot or root (be especially careful with longer roots, as they can easily bend and break). Plant it into the gap you made in the agar such that the root reaches beneath the agar to where the *Striga* seeds are embedded and that it points toward the center of the plate. The kernel should be at least 1 cm from the edge of the plate. Use the forceps to make the split in the agar close over the remaining root, again being very careful not to damage it. Done properly, the root will grow across the plate among the *Striga* seed and the shoot will grow in the space between the top surface of the agar and the lid. Prepare at least three plates per sorghum accession being tested, more if the accession is heterogeneous (like a landrace or segregating population). I prefer to prepare four plates of each from which the best three best will be used for measurements. Wash forceps in 100% ethanol between each accession. Leave one plate with the same batch(es) of *Striga* blank (no sorghum) to test the germinability of each *Striga* batch. After all accessions are plated, move in stacks of three on trays to shelves in the 29 °C incubator where they will remain for 3–4 days. After returning other plates to the incubator, open the blank plate (no sorghum) and lean it against the back of the hood. Get the atomizer containing 10^{-5} M GR24 (that you removed from the 4 °C earlier, it should now be at room temperature, see inset "*How to prepare and keep a clean and active GR24 solution for applying to agar*

plates"), swirl it to mix and spray the exposed agar with two firm squeezes (three if you are spraying a 25 mm deep plate) of the bulb. Make sure you are holding the spray bottle upright so that the bottom of the atomizer reservoir is parallel to the surface of the transfer hood when you squeeze the bulb. This should deliver around 35 μL of the solution so that once it diffuses through the 35 mL (or 60 μL to 60 mL in deep plates) of agar, gets diluted to around 10^{-8} M. Return spray bottle to the 4 °C refrigerator. Replace lid on the sprayed blank plate and return to incubator, stacking it along with those planted with sorghum no higher than three plates to a stack. Check the plates in three days. As long as the roots of the sorghum have grown across the plate, most being *at least* 5 cm long, they are ready to read.

Reading Striga *germination at Day 3*. With the blank plate(s), that is a plate run in the same batch as the ones being read that contains only *Striga* with no sorghum sprayed with GR24, randomly position the plate in at least ten different areas under a dissecting microscope, counting both the number of *Striga* seeds and the number of germinated *Striga* in individual 2 × 2.5 cm fields. If the *Striga* seed density is ideal, you will be counting about 100 seeds in each 5 cm^2 field. These counts may be taken later by imaging the ten fields. Observe all plates through the bottom of the plate because the root and *Striga* seed are closest to this surface and you won't have to look through the layer of agar. Record each of these counts on the data sheet. Aim at counting around 1000 seeds. Calculate the *Striga* batch germination rate for this set of plates by adding all the counts (from each of the ten field counts) for no. germinated *Striga* and dividing this by the total number of *Striga* seeds (in the ten fields). This should be at least 30%. If it is less, the *Striga* seed is not responsive enough to give you a meaningful measure of *Striga* germination stimulant activity on your sample (sorghum) plates and so you shouldn't waste more time reading those.

Provided you have > 30% germination on your blank plates, the plates containing sorghum are checked under the dissecting microscope after 3 days. Before taking them to the microscope, wipe any condensation inside the plate lids with clean paper towels (plates should only be opened under the transfer hood). Select the best three plates out of the four prepared for each entry (more if you're looking at a heterogeneous group) for microscopic observations. Selections should be based on health of the host plant and whether its root has properly penetrated the agar. Assign each a number (− 1, − 2, − 3) and mark it on the top and bottom of each plate next to the entry number marked when planting the sorghum. Record the condition of each host plant on the data sheet (Fig. 5). Use the descriptor "G" (good) for normal looking sorghum or "F" (fair) if the root or shoot is especially small or there is some other problem. Record any problems with host condition in the notes column of the data sheet. If the sorghum seedling is damaged or didn't grow, and its main root is < 3 cm long, return it to the incubator if you have less than three plates for that entry. Check it again in a day or two and use it only if the root exceeds 3 cm.

Observe plates at about 10× magnification (again through the bottom of the plate). Look for germinated *Striga* along the sorghum root beginning 2 cm from the kernel. Mark a line at the basal 2 cm and only search for germinated *Striga* beyond this mark (toward the root apex). Mark a small dot on the plate over the seed coat of the three germinated *Striga* furthest from each host root (Figs. 6 and

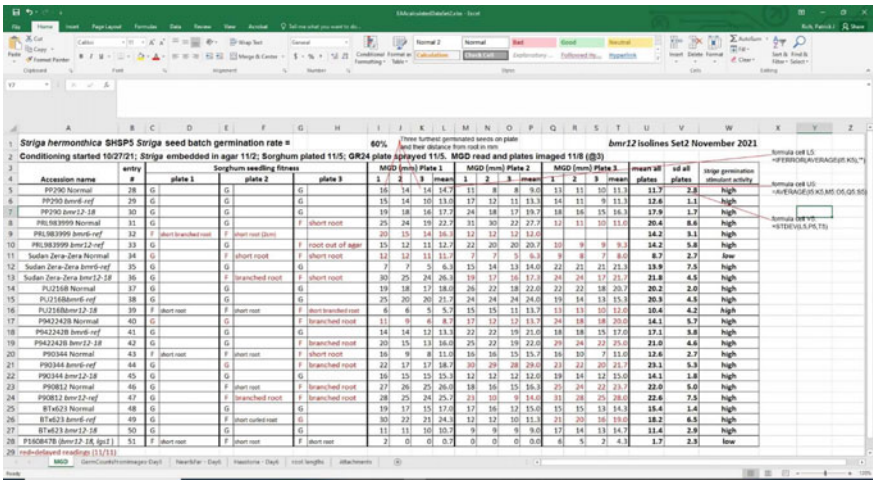

Fig. 5 Example data sheet for recording MGD

7). Try to avoid areas within 2 cm of the kernel if the main root happens to curl back toward it. Using a transparent ruler, record the distances of these marks (to the nearest 0.5 mm) to the nearest sorghum root (main root or branch). If less than three germinated *Striga* are seen, record this on the sheet. Maximum germination distance (MGD) is the average of these three measurements on each seedling. If less than three germinated weed seeds are observed, enter "0" in the calculations for average. For example, if you observe only one germinated *Striga* in the measuring zone and the distance from that to the nearest root is 1 mm, then the MGD is 0.3 mm [(1 + 0 + 0)/3]. MGD for the accession is the average of at least three good seedlings. Also calculate the standard deviation between seedling MGDs of a given accession to give an indication of the variability between them. The MGD is a measure of *Striga* germination stimulant activity. Generally, a sorghum seedling with an MGD > 10 mm has high *Striga* germination stimulant activity. A seedling with an MGD < 5 mm has low *Striga* germination stimulant activity. Those with MGDs between 5 and 10 are borderline lows and may indicate influence of germination inhibitors (see next section). All measures should be considered in the context of the standard high and low checks run with the set of plates. Sometimes the 10 mm threshold will need adjusting slightly, particularly for those borderline accessions or with low responsive *Striga* seed sources. A sorghum line truly having the resistance mechanism of low *Striga* germination stimulant activity should consistently show MGDs below 10 mm from set to set.

Some optional measures—extending the agar assay. Near host germination rates can also be recorded at Day 3 to get another measure of *Striga* germination stimulant activity. A standard area is selected for all observed seedlings so that comparisons of

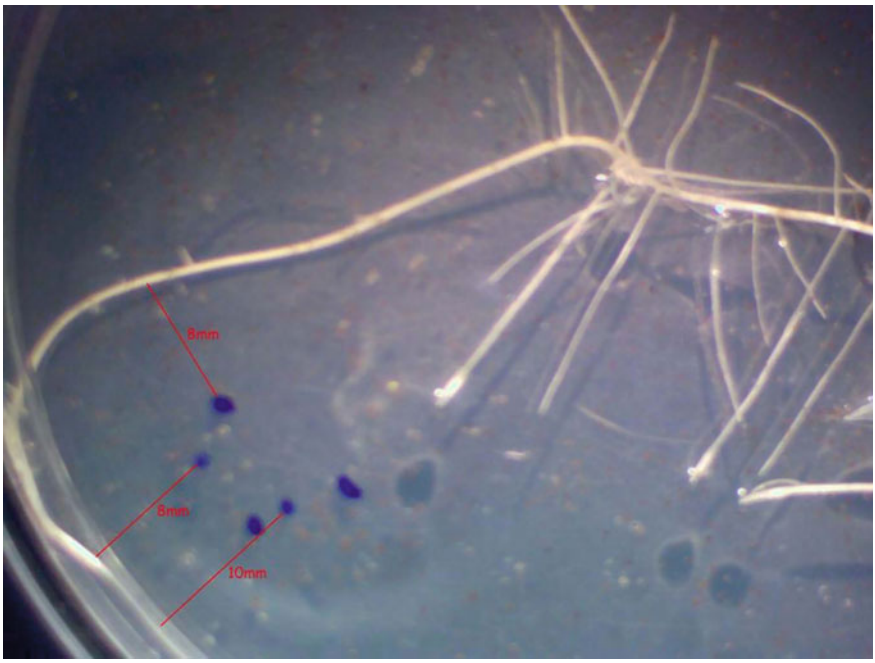

Fig. 6 Example of MGD measures at Day 3. Blue dots were marked on bottom of plate on what appear to be the furthest germinated *Striga* seeds. Distances of the three furthest to the nearest root are measured and averaged for the MGD [in this case, $(8 + 8 + 10)/3 = 8.7$ mm]

Striga germination stimulant activity as well as any possible germination inhibition and influence on haustorial formation can be made. This measure generally correlates well with MGD except in cases where germination inhibitors are present in the sorghum root exudate. It is also useful for comparing to in vitro germination assays using collected sorghum exudates. To prepare for these counts, cut a template from a transparent ruler that is 2×2.5 cm. Use this or another ruler to make a small mark 2 cm from the sorghum kernel along the main root. Use the template to trace a rectangle on the bottom of the plate, keeping one of the narrow edges (a 2 cm side of the rectangle) on the 2 cm mark along the main root you made from the kernel. Try to keep the main root in the center of this rectangle so that it splits it in half, that way the rectangular space you will observe under the microscope will cover a 5 cm^2 area along the main root from 2 to 4.5 cm from the kernel and roughly 1 cm on either side of the main root. After marking all your plates with these rectangles, look at them each under the microscope, setting the magnification to around the 1.4 mark, or there about, such that the edges of your drawn rectangle match the edges of your viewing field. Once you've set this field of view for the first plate and focused and set the illumination to clearly see the main sorghum root and surrounding *Striga*, you shouldn't have to change it for the other plates. Snap a photo of each plate, keeping track of which image goes with which plate. Make sure you always orient each plate

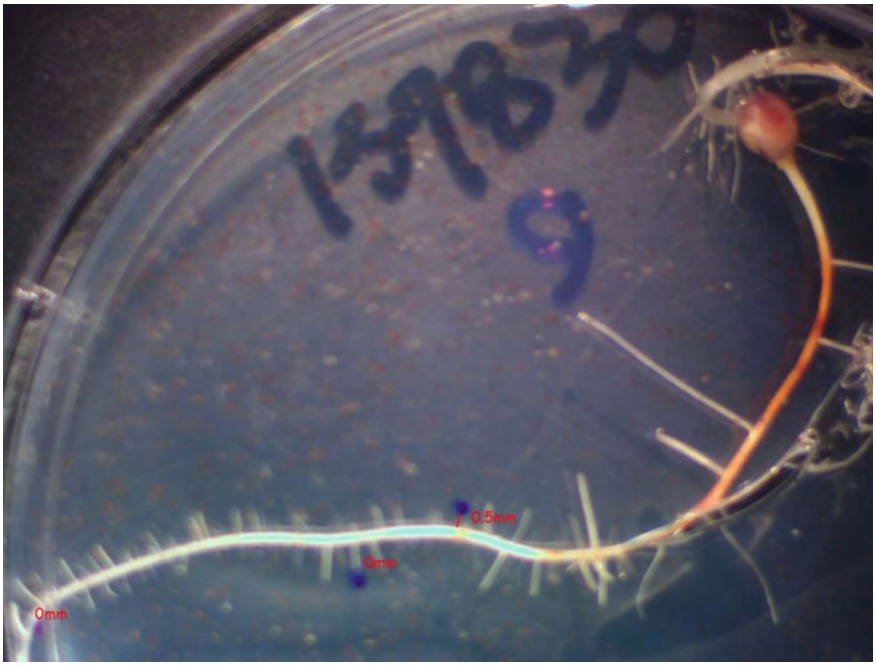

Fig. 7 Another example of MGD measure at Day 3, this time on a sorghum seedling with low *Striga* germination stimulant activity [$(0 + 0 + 0.5)/3 = 0.2$ mm]

as you image them so that the basal end of the main root (toward the kernel) is always to the right of the captured image. These images contain all the *Striga* within about 1 cm on each side of the main root in this zone and from these counts of germinated and total number of *Striga* seed can be made later. Record these on the data sheet (Fig. 8). Because germinability varies between conditioned batches of *Striga* seed, it is a good idea to correct germination rates counted in your images by dividing the germination rates by the batch germination rate determined from the blank plate that you treated with GR24 at Day 0 and from which you imaged 20 random 5 cm^2 fields on Day 3. This "corrected" germination rate allows you to more meaningfully compare plates run in separate batches or with different *Striga* seed sources, as it tells you the proportion of viable seeds that responded to the sorghum root germination stimulants.

To overcome differences in *Striga* germination stimulant activity between sorghum accessions for post-germination measures, you can spray all the plates (after imaging) with 10^{-5} M GR24 (final conc. = 10^{-8} M, after diffusing through the agar) as you did earlier for the blank plate, under the hood and return them to the incubator for two more days.

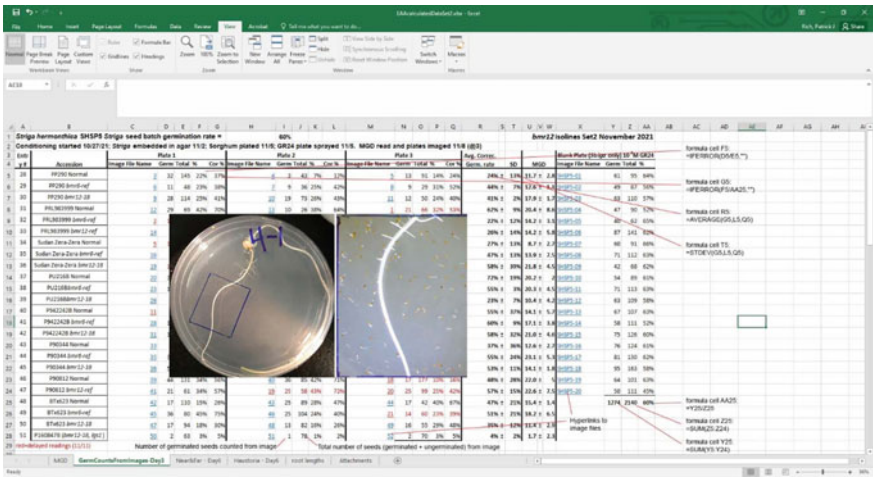

Fig. 8 Example data sheet for recording Day 3 measures of germination rates. Germination rates are corrected for germinability of *Striga* seed source used

How to prepare and keep a clean and active GR24 solution for applying to agar plates

1. Prepare a GR24 "stock solution"

Dissolve 0.024 g GR24 (a synthetic strigolactone, molecular wt. = 298.3) in 10 mL DMSO

0.024 g/0.01 L = 2.4 g/L $\approx 8 \times 10^{-3}$ M (in 100% DMSO)

Keep this frozen in a brown glass bottle at − 20 °C when not in use. Thaw thoroughly before using. Stored properly, this solution will last several years

2. Prepare the GR24 "application solution"

Add 250 μL "stock solution" to 100 mL ddH_2O in a clean brown glass bottle

8×10^{-3} M GR24 × 0.25 mL/100 mL = 2×10^{-5} M (in 0.25% DMSO)

Use this solution to fill the atomizer (15-RD glass atomizer with plastisol bulb, DeVilbiss Healthcare LLC) which holds about 25 mL. Keep the remainder (not put in the atomizer) frozen at − 20 °C when not in use. Thaw thoroughly before filling the atomizer and shake after thawing to redissolve any GR24 which may have precipitated. Keep atomizer in a refrigerator (4 °C) when not in use. Bring to room temperature before spraying plates

3. Spraying the plates

Open agar plates only under a laminar flow hood to avoid surface contamination. GR24 is usually applied after measuring MGD and imaging for germination counts 3 days after plating the sorghum in the agar embedded with Striga.

Only open a few plates at a time when spraying to avoid drying them out under the airflow of the hood. Lean them at the back of the hood so that the surface of the agar is perpendicular to the work surface. Take care not to damage the sorghum shoot which may fall from the bounds of the plate edges. Holding the glass reservoir of the atomizer in one hand such that the delivery nozzle is 10–15 cm from the exposed agar surface, give the bulb two firm squeezes with the other hand. This should deliver about 35 μL (≈ 17 μL per squeeze) to the agar. If you are using 25 mm deep plates containing 60 mL agar, spray with three firm squeezes of the bulb. It is important to keep the bottom of the atomizer reservoir parallel to the work surface of the hood. Tilting it excessively will not deliver the proper volume. Assuming the volume of the agar is 35 mL (60 mL in deep plates), the final concentration of GR24, once it diffuses through to the embedded Striga seed, will be in the range of 10^{-8} M, which is ideal to germinate all germinable weed seed. The final DMSO concentration is negligible and will not harm the plants. Replace the lids on the sprayed plates promptly, returning them to a horizontal position. Carefully guide the sorghum shoot back into the confines of the plate if it came out during spraying. Make sure not to pinch it between the bottom plate sides and lid. Wait two days before imaging for post-GR24 germination counts. Return atomizer to refrigerator. A full atomizer reservoir should be enough to spray 100 plates

Two days after spraying the plates with GR24 (Day 5), mark a second 2 × 2.5 cm rectangle on the bottoms of the plates using the template you used on Day 3 (Fig. 9). This time, however, choose an area as far from any roots (especially the main root) as possible on the plate, avoiding that area within 2 cm of the kernel. Use a different color for this rectangle than what was used on Day 3. Where you place this second rectangle will vary from plate to plate, the idea being that you observe *Striga* seeds minimally influenced by any sorghum roots so that you can compare these to that *Striga* within 1 cm on each side of the main root from 2 to 4.5 cm. Since you sprayed the plates with GR24, most weed seed on the plate, whether near or far from the sorghum root, ought to have received adequate germination stimulant. Reimage the rectangle you marked on Day 3, the "near-host-root", and snap another image of the new "far-from-root" 2 × 2.5 cm area for each plate. Again, keep the plates oriented in the same way (kernel to the right) as you did on Day 3 and keep track of each image file name with your "near and far" images of each plate.

By comparing these areas, in terms of *Striga* germination rate particularly, you can measure any inhibition that a sorghum root might have on *Striga*. Germination counts are made on your Day 3 and Day 5 images by dividing the number of germinated *Striga* seeds in the image by the total number of seeds and express as a percentage to determine the germination rate. You may also want to determine the percent germinated *Striga* near the root with haustoria at both Days 3 and 5. To do this, count the number of germinated *Striga* with haustoria. Only those *Striga* radicles that are

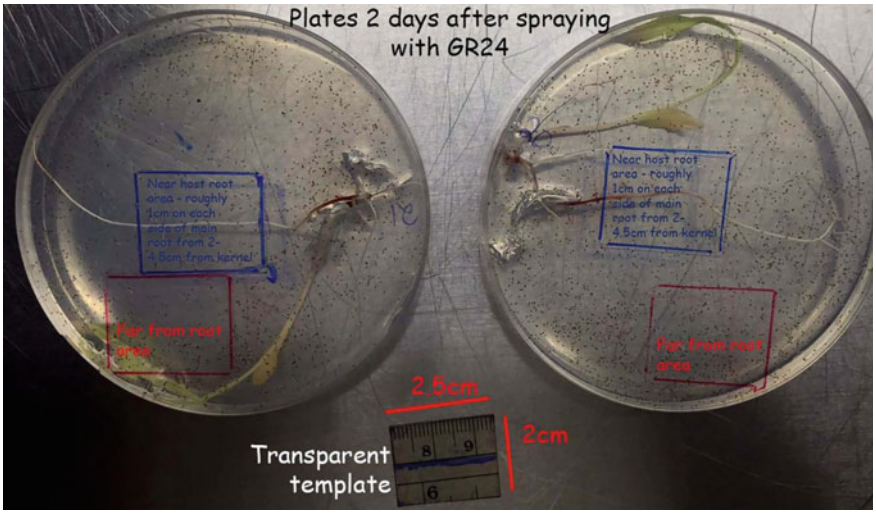

Fig. 9 Agar gel assay plates marked for "Near & Far" measures. After reading the germination distance (MGD) at Day 3 and imaging the area 2–4.5 cm from the kernel for observations and germination counts (blue rectangles), plates are sprayed with 35–40 μL of 10^{-5} M GR24 (final conc. = 10^{-8} M) to germinate remaining *Striga* seed on plate that was not stimulated by the host root and returned to the incubator for two more days. At Day 5, before reimaging the "near" area (blue rectangle), a second (red) rectangle is drawn on the plate as far from the main root as possible tracing the same 2 × 2.5 cm template cut from a transparent ruler (bottom). Both areas are then imaged and germination rates within each compared for any indication of germination inhibition

wider at their apical end relative to their basal width are counted as having haustoria. Divide this count by the number of *germinated* weed seeds in the area (Fig. 10).

To determine if there is any germination inhibition near the host root, use the two images for each plate taken after GR24 treatment, the Day 5 images. Count the number of germinated seeds and total seeds in each rectangle to determine the germination rates in each place. Then divide the germination rate calculated for the "near-host-root" area by the germination rate of the "far-from-root" area to obtain the "germination index" (GI). To avoid effects of peculiarities on certain plates where root-free areas are hard to find, average the far-from-host germination counts for all plates in a set (all plates from all entries tested in that set) and use this value as the germination count by which to divide each near-host-root rates to determine the GI. Average these germination indices for all plates observed for a given accession (Fig. 11). Example images from which these counts are taken are shown in Figs. 12 and 13. A GI wildly different than one could indicate some inhibition of the sorghum accession on *Striga* germination. Most sorghum does show some germination inhibition by this measure, that is, GI in the range of 0.7–1.0. Sorghum accessions that consistently show a GI $\leq$ 0.65 in this assay, especially with multiple *Striga* seed sources, we consider to have a germination inhibition mechanism of *Striga* resistance. The impact of this trait on overall resistance and its chemical nature remains to

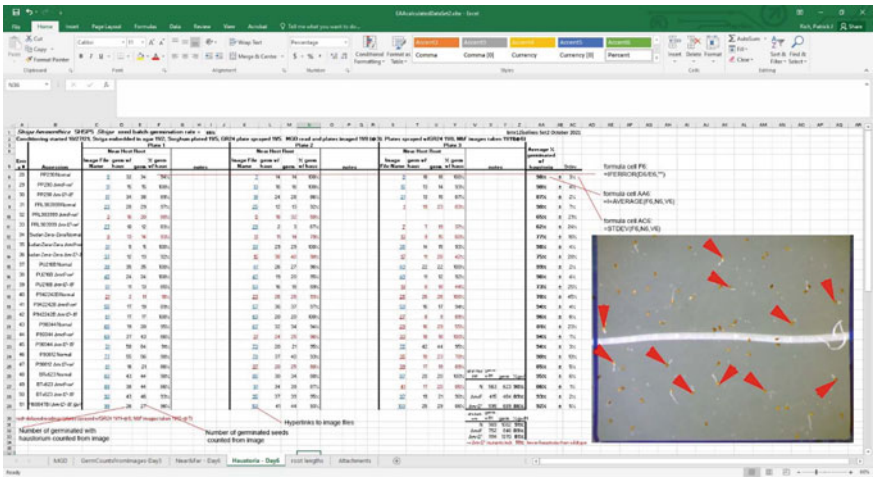

Fig. 10 Example data sheet for recording Day 5 proportion of germinated *Striga* forming haustoria. Red arrows on example image indicate germinated *Striga* with haustoria

be determined. It is, however, clearly distinct from low *Striga* germination stimulant activity.

You may also note some other allelopathic influences of certain accessions on surrounding *Striga* in the Day 5 "near" images. Do haustoria form in germinated *Striga* at similar rates? Do these haustoria look unusual? Do the *Striga* come out of their seed coats? Are *Striga* radicals unusually short or opaque near the sorghum

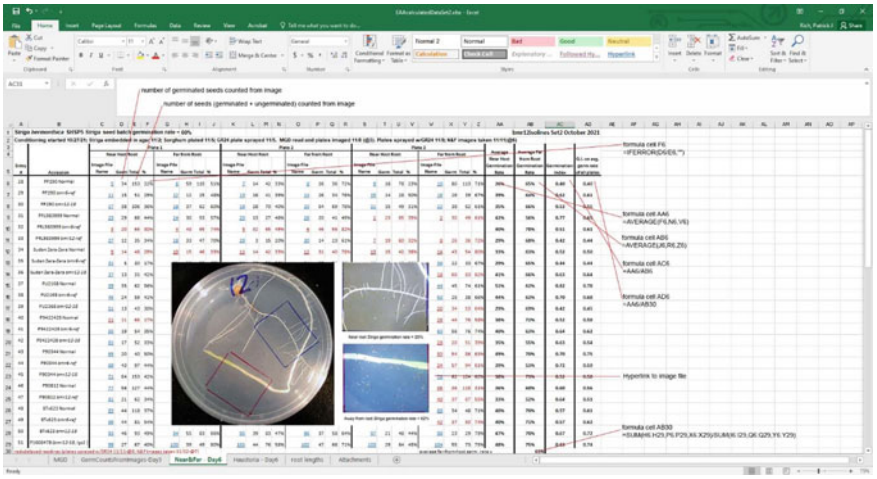

Fig. 11 Example data sheet for measuring germination inhibition

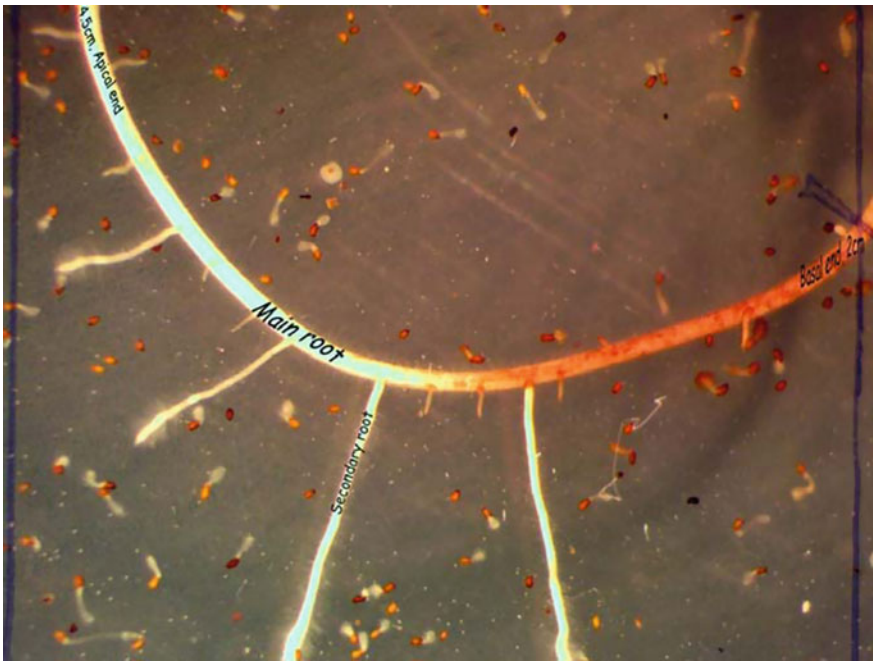

Fig. 12 Image of "near-host-root" area. Image taken at Day 5 (2 days after GR24 treatment) covering an area of 2 × 2.5 cm. This includes *Striga* within approximately 1 cm of the host main root over a length of 2 cm (right) to 4.5 cm (left). Note that most germinated *Striga* in this area have haustoria. Toward the basal end (right) an orange colored exudate can be seen being absorbed by surrounding *Striga*

root? Do you notice colored exudates (yellow, orange or red) bleeding from the sorghum root and being absorbed by the surrounding *Striga* (Fig. 12)?

You may also want to count the number of lateral branches on the sorghum root, their lengths, etc. as an indicator of rooting habit. These kind of measurements are easier if you can get a digital image (or images) of the entire plate. If the microscope you have won't quite zoom out that far, a smart phone camera using the "square" setting is a good way to image the entire plate. Even better if you can compare two images of the same root, perhaps at Day 3 and Day 5, to get an idea about root growth rate over that specific time. Software that comes with some microscopes or root analysis software like ImageJ® (available for free from the United States National Institutes of Health at https://imagej.nih.gov/ij/) can assist with such measures.

Even more from the agar plates. If you use deeper (25 mm) plates with thicker agar layers (60 mL) you should be able to keep the plates for observing any *Striga* attachments on the sorghum seedlings. Leaving the plates stacked only two or three high on a lab bench under fluorescent lights can keep the sorghum seedlings alive and growing for three weeks. As long as the plate remains free from mold or bacterial contamination and the sorghum shoot remains green, plates remain useful. Wiping

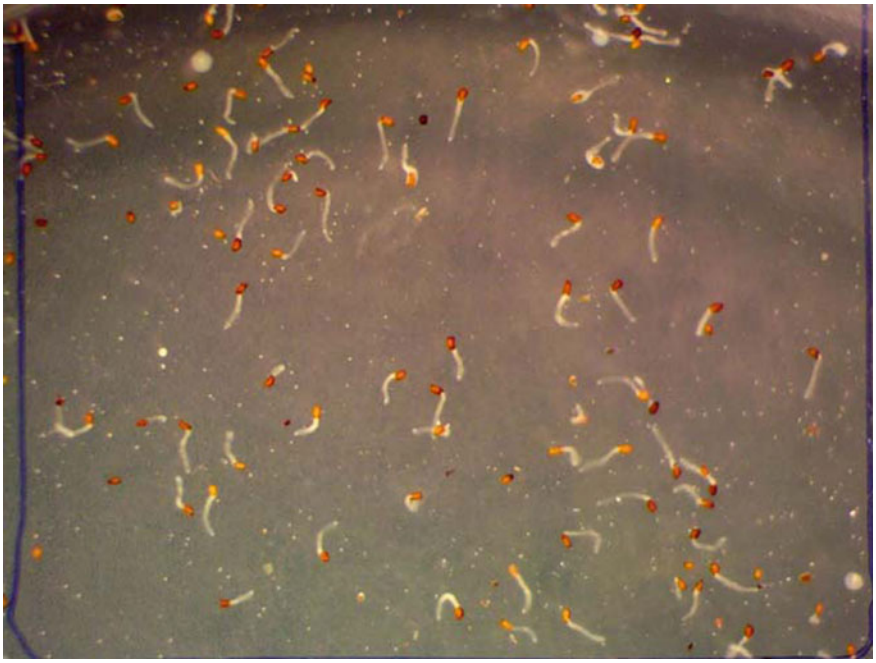

Fig. 13 Image of "far-from-root" area from the same plate. Image taken at Day 5 (2 days after GR24 treatment) from the same plate covering a 2 × 2.5 cm area away from the sorghum root. Note germinated *Striga* here lack haustoria since they are not near enough to the host to receive the haustorial initiation signal

out condensation inside the lid of the plate where the sorghum shoot touches also helps to keep them going. If, after 10 days from when you planted the sorghum in the agar, the sorghum shoot is green and the agar layer hasn't dried out (still comes to at least the half full mark in the bottom of the plate), you can check them under the microscope for attachments. With the magnification set in the mid-range, follow the main root and its branches to look for *Striga* that have attached. Circle and mark each with a unique number on the bottom of the plate and take a picture of each at higher magnification. The position of each on the root system (whether they are on the main root or a branch, for instance) can be recorded from a whole plate image taken with a smart phone at each observation day. If you can get two or three images over time of particular attached *Striga* on a live host, you can have a measure of its ability to support early *Striga* growth. Imaging individual attachments marked at 10 days and reimaging at 15 and 20 days (or there about) after planting the sorghum allows one to get an idea of whether and how well *Striga* grow on a particular sorghum accession. Simple data collected from tracked individual attachments as "successful" if the parasite grows over the course of observation or "unsuccessful" if an attached *Striga* does not grow allows one to compare sorghum accessions both in terms of the average number of attached parasites per seedling and percentage of those that

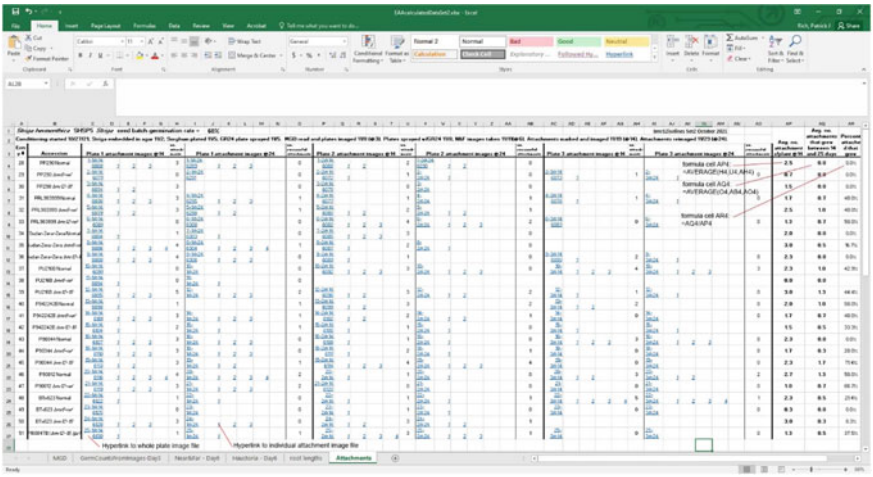

Fig. 14 Example data sheet for keeping track of *Striga* attachments and quantifying post-attachment resistance in sorghum in agar plates

succeed (Fig. 14). Such measures can indicate differences in their compatibilities as *Striga* hosts. With the proper checks, the extended agar assay in 25 mm plates can be used as an initial screen for post-attachment *Striga* resistance.

It is very important that you keep track of the fitness of the sorghum on the plate with each set of images and make sure the agar hasn't dried out if you go to the trouble to follow the growth of individual parasites over time (Fig. 15). Whole plate images are useful for this purpose. All *Striga*, even on a susceptible host, is going to look sick and die if its host is dying or dead. Don't bother looking at plates with a sorghum whose shoot is brown or on which the agar layer is drying up. Also your record keeping needs to be such that you know which image corresponds to which *Striga* on which plate so that you can put together a string of images at the end of the observation period (perhaps 3 images over 2 weeks, Fig. 16). This is, of course, laborious and probably not worth it if your attachment frequency is low. Attachment frequency tends to be higher in thicker agar layers (60 mL) in deep (25 mm) plates. Resistance at this early post-attachment stage, generally called incompatibility, may be observed if you have several good plates of a given accession. If a sorghum accession consistently shows fewer attachments than a susceptible check in the same set, this may indicate some post-germination, pre-attachment resistance. If the *Striga* attaching to plants of one accession don't grow, turn purple or grows at a slower rate than those on a susceptible check, you might be observing incompatible resistance (Fig. 17).

The agar assay is not ideal for measuring these post-germination resistance mechanisms because the sorghum seedling is so confined and attachment frequency tends to be lower in agar than on other media like that described in Chapter "Phenotyping for Resistance to *Striga Asiatica* in Rice and Maize Mutant Populations In Madagascar". With several sets of good plates with multiple *Striga* attachments and careful

Fig. 15 If given proper light and humidity and a thick enough layer of agar, it's possible to keep plates for three weeks or even longer. Even in this set of standard depth (15 mm) plates, young *Striga* can be seen growing on 20-day-old sorghum seedlings. The sorghum eventually succumbs to the cramped conditions and lack of nutrients

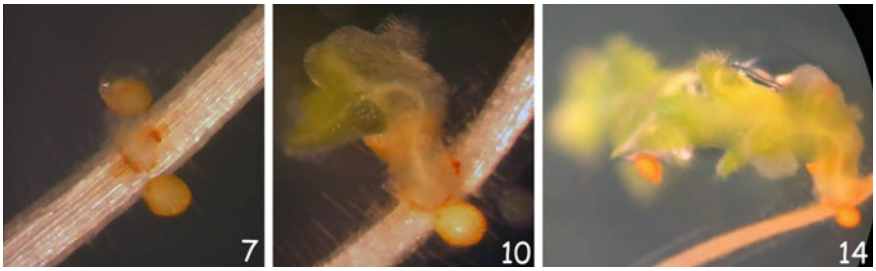

Fig. 16 Images of the same *Striga* attached to the root of a susceptible sorghum over time in agar. Images taken at 7, 10 and 14 days after planting the sorghum seedling in agar embedded with conditioned *Striga* seed. Plate was treated with GR24 after reading MGD in the Agar Gel Assay. In this case, images at the latter days had to be taken at lower magnifications than the original image to capture the larger *Striga* shoot

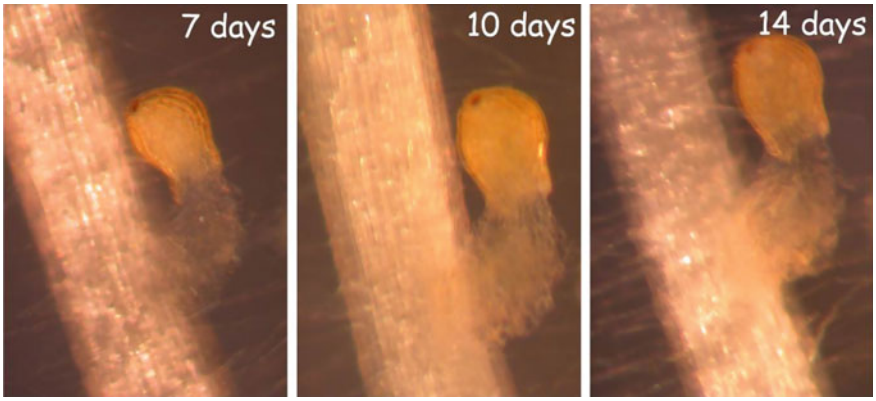

Fig. 17 Images of the same *Striga* attached to the root of a resistant sorghum over time in agar. Images taken at 7, 10 and 14 days after planting the sorghum seedling in agar embedded with conditioned *Striga* seed. Plate was treated with GR24 after reading MGD in the Agar Gel Assay. In contrast to the series of images collected on the susceptible sorghum, this attached *Striga* does not grow

observations, such reactions can be indicated from the same plates used to determine if pre-attachment *Striga* resistance mechanisms are at play. Post-attachment *Striga* resistance can then be confirmed in other co-culture media.

Conclusions

The most important information (and what the assay was originally designed for) one gets from the agar gel assay is the MGD, which is an established measure of *Striga* germination stimulant activity (Hess et al. 1992). As long as one has the proper

amount of *Striga* seed embedded in the agar, and the germinability of that seed is adequate (> 30%, as measured on the blank plates), one can determine whether a sorghum accession has the resistance mechanism of low *Striga* germination stimulant activity. To date, the only *Striga* resistance gene identified in sorghum is *LGS1* which controls germination stimulant activity (Gobena et al. 2017). Recessive alleles at this locus changes the type of strigolactone exuded by sorghum roots. Mutagenesis could result in new *lgs1* alleles which could be confirmed in mutant lines by chemical analysis of root exudates (This chapter) and sequencing of PCR amplicons targeting *LGS1*. More useful would be gained resistance expressed as germination inhibition, lower haustorial inducing capacity or at the post-germination stages of the *Striga*-host interaction. Then *lgs1* could be introgressed into these mutant lines through marker assisted backcrossing to offer more durable *Striga* resistance through pyramiding. The extended agar assay may detect such novel resistance mechanisms and ultimately, with further investigation, lead to gene identification underpinning the gained *Striga* resistance.

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Histological Analysis of *Striga* Infected Plants

Mafrikhul Muttaqin, Songkui Cui, and Satoko Yoshida

Abstract Post-attachment resistance can be verified by observing the infection and growth rates of *Striga* attached to host roots. These protocols explain how to infect *Striga hermonthica* on host roots using rhizotron chambers, a semi-in vitro system that allows one to observe *Striga* infection without disturbing the host's roots. Sectioning parasitic attachments and subsequent histological methods are also described, which allow closer investigations of the extent to which the *Striga* haustoria penetrate host tissues. Such observations can reveal whether any mechanical resistance exists in the host. These histological methods help further characterize resistance mechanisms in rice or sorghum.

Keywords *Striga* · Root parasitic plants · Rhizotron · Safranin · Xylem staining · Histology

Introduction

Striga resistance can be classified into two types, pre-attachment and post-attachment. As *Striga* parasitism advances on its cereal hosts, pre-attachment processes include germination and haustorium formation, whereas post-attachment processes include penetration, reaching the host stele, xylem connection, and nutrient transfer. To characterize any gained *Striga*-resistance in mutagenized hosts, it is essential to quantify and qualify the resistance. For qualification of *Striga* parasitic success, developing a system in which multiple *Striga* synchronously infect host roots with minimal influence from environmental conditions are key. For this purpose, we

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use a rhizotron system, in which host roots infected by *Striga* are easily observed without disturbing root architecture (Gurney et al. 2006; Yoshida and Shirasu 2009). The rates of infection are quantified with low-power stereo microscopy or magnification lenses at different days of co-culture. When a *Striga* seedling has more than three pairs of leaves, the vascular connection generally is established (Yoshida and Shirasu 2009). To characterize the resistance mechanisms in detail, histological analysis is useful. The xylem bridge can be observed with simple Safranin O staining, and the cellular level interaction can be analyzed by making thin sections from Technovit 7100-embedded samples. The staining process may then reveal some post-attachment resistance mechanisms (Yoshida and Shirasu 2009), including incompatibility after vascular connection, endodermis blockage, or mechanical barriers to penetration at the root cortex.

Protocols

Rhizotron system for observing Striga *infection.* Although the rhizotron method is described for rice, it is also suitable for use with a sorghum host. If using sorghum, follow the surface sterilization and germination protocol described in Chapter "An Agar-Based Method for Determining Mechanisms of Striga Resistance in Sorghum".

Surface sterilize rice seeds by soaking in a tube with 10% commercial bleach (0.525% NaOCl) for 15 min, gently vortexing periodically. Rinse five times with sterile water and then sow on a Whatman GF/A filter paper in a 90 mm petri dish filled with 15–20 ml sterile water. Germinate the seeds and grow for one week in a growth chamber at 26 °C with the 16 h light/8 h dark photoperiod.

Assemble the rhizotron by removing a 2 cm square notch on the shorter side of a square petri dish and its lid using a pair of pliers or a soldering iron. If using a soldering iron, work under a fume hood to remove fumes from melting plastic (Fig. 1). Make two drain holes on the opposite short side of the rectangular petri dish using the soldering iron (Fig. 1). Cut the rockwool and nylon mesh to the same dimensions of the bottom plate of the petri dish. Immerse the rockwool in a tray filled with tap water. Place the rockwool into the petri dish, and cover with nylon mesh. Place the rice seedling on the nylon mesh. Close the petri dish with its lid and secure it with two rubber bands. Place the petri dish vertically on a tray with water, cover the root parts with aluminum foil, and give half-strength MS for rice plants. Grow them in a glasshouse or growth chamber (28 °C light for 12-h/20 °C dark for 12-h).

Preparation of Striga *seeds (preconditioning).* At 1–2 weeks before infection day, surface-sterilize *Striga* seeds by immersing them in a 20% commercial bleach solution diluted to contain approximately 1.2% sodium hypochlorite (NaOCl) for 5 min. Replace the solution at least once, wait for 5 min, and wash with sterile water more than five times. Place the sterilized seeds on the glass microfiber filters GF/A paper with 8–10 ml water in a petri dish (90 mm diameter), cover it with aluminum foil, and keep it in a 25 °C plant growth chamber for 1–2 weeks.

Fig. 1 Materials and structure of a rhizotron

Striga *infection.* Before *Striga* infection, add germination stimulants (final concentration of 10 nM strigol, or 1 μM GR24) onto the filter paper with preconditioned *Striga.* Return the petri dish at 25 °C and incubate for 2–24 h in the dark. Open the rhizotron and place the *Striga* seeds carefully along the rice roots using forceps (5–10 mm distance between seeds, Fig. 2). Put the rhizotron lid back and secure the rhizotron with two rubber bands. Cover the root parts with aluminum foil. Place it back to the glasshouse or plant growth chamber. In the case of two hours exposure to germination stimulants, *Striga* germination can be observed at one day, penetration at two days, and xylem bridge formation at 3–5 days after infection. In the case of 24 h germination stimulant treatment, *Striga* germination can be readily observed before rice infection, and penetration and xylem bridge formation can be observed at one day and 3–4 days after infection, respectively.

Quantification of Striga *infection.* After a few weeks of infection, *Striga* infection rates can be quantified depending on which stage you want to observe. The later stage can be analyzed at two and four weeks after infection. Carefully remove the rubber bands and the lid from a rhizotron without disturbing host roots. Observe the infection stage of each *Striga* seedling under a stereo microscope or magnification

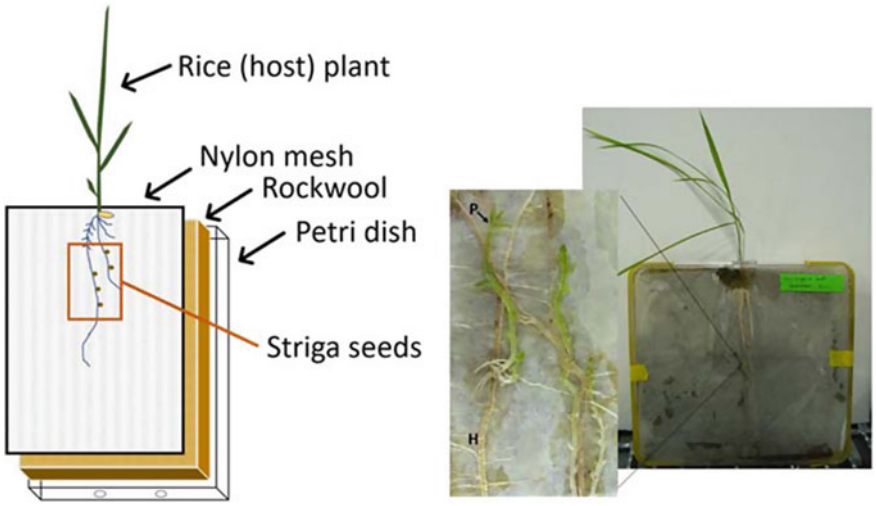

Fig. 2 Rhizotron infection system; P: parasite (*Striga hermonthica*), H: host (rice)

lenses. Count the number of *Striga* in each stage. *Striga* infection can be classified into the following seven groups, (a) not germinated, (b) germinated but not attached, (c) penetrated, but the seed coat is still covered, (d) penetrated but dead, (e) 1–2 pairs of open leaves, (f) 3–5 pairs of open leaves or (g) more than six pairs of open leaves (Yoshida and Shirasu 2009) (Fig. 3).

Safranin O staining for the observation of xylem bridges. Prepare a fixation solution by combining 75% ethanol, 25% acetic acid, and 0.1% Safranin O. Prepare a clearing solution by dissolving 1 g chloral hydrate ($C_2H_3Cl_3O_2$) in 1 mL glycerol and 2 mL water.

Excise 1–2 cm of *Striga*-infected root segments with scissors. Place root samples in the fixation solution and vacuum infiltrate for 15 min. Wash with sterile water three times. Add enough 0.1% Safranin O stain to cover the sample and heat at 90 °C for 5 min. Wash with sterile water three times. Soak in the clearing solution overnight. Observe the xylem bridge formation under microscopy. Xylem cells are stained in red with Safranin O (Fig. 4). The staining can also be observed under fluorescent microscopy with the filter setting for red fluorescent protein (RFP).

Making Technovit 7100-embedded thin sections. Prepare an FAA fixative solution by combining 1 mL formaldehyde, 0.5 mL glacial acetic acid, 5 mL ethanol, and 3.5 mL water. Prepare the following steps at room temperature. Cut the samples and put them into the FAA fixative solution, vacuum infiltrate for 15 min and leave at room temperature for more than two hours (place at 4 °C if you do it overnight). Dehydrate using an ethanol series. Replace fixative solution with 70% ethanol and incubate for 30 min with gentle agitation at room temperature. Next, replace 70% ethanol with 100% ethanol and incubate for 30 min with gentle agitation at room temperature. Repeat 100% ethanol incubation one more time.

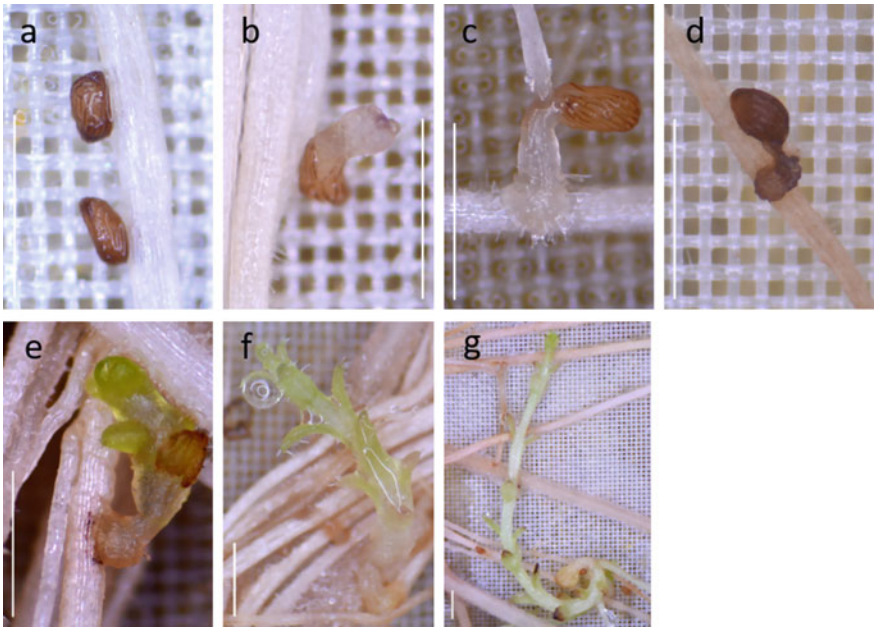

Fig. 3 Classification of *Striga hermontica* infection on a sorghum host. **a** Not germinated, **b** germinated but not attached, **c** penetrated, but the seed coat is still covered, **d** penetrated but dead, **e** 1–2 pairs of open leaves, **f** 3–5 pairs of open leaves, and **g** more than six pairs of open leaves. Scale bars: 1000 μm

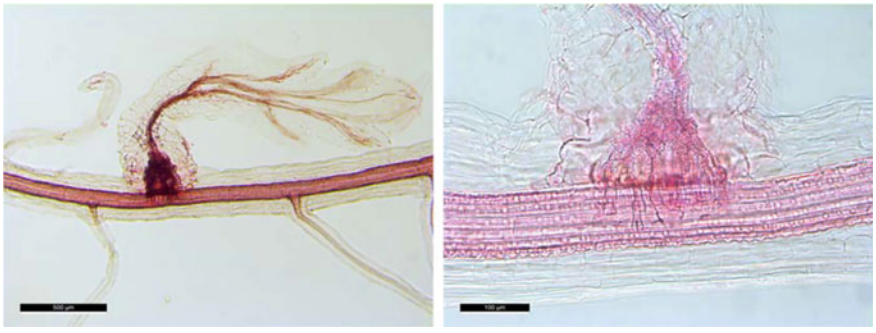

Fig. 4 Safranin-O staining of *Striga hermonthica* infecting a sorghum root. Scale bars: left 500 μm, right 100 μm

Add 1/4 volume (250 μL for 1 mL ethanol) of Technovit 7100 liquid (Heraeus Kulzer GmbH; liquid, Hardener I, Hardener II), and incubate for 30 min with gentle agitation. Repeat until the solution contains an equal volume of Technovit 7100 liquid and ethanol (four times). Incubate for more than one hour (or overnight) with gentle agitation. Remove half the volume of Technovit 7100 liquid: ethanol

solution. Add the same volume of Technovit 7100 liquid as you removed. Incubate for 30 min with gentle agitation. Prepare Technovit infiltration solution (add 1 g of Hardener I to 100 mL Technovit 7100 liquid). Replace the solution containing the sample with the Technovit infiltration solution. Incubate it for more than one hour with gentle agitation. Replace the solution with a new Technovit infiltration solution. Incubate it overnight with gentle agitation. Replace the solution with a new Technovit infiltration solution. Incubate it for 1–2 h(s) with gentle agitation. Prepare the Technovit embedding solution by mixing 1 mL Technovit infiltration solution and 66 μL Hardener II (this will start solidifying soon).

To embed the infiltrated *Striga*/root sample, cut it under a microscope to the appropriate size and shape. Prepare a proper mold (e.g., cap of 1.5 ml Eppendorf tube, etc.). Put the sample into the mold and add the Technovit embedding solution. Adjust the angle of samples. Leave it at room temperature (RT) until the sample becomes solid (1 h at RT or 37 °C, or overnight at 4 °C or RT).

Prepare a Technovit 3040 (Heraeus Kulzer GmbH; powder, liquid) mounting solution by mixing well Technovit 3040 powder and liquid at a 2:1 ratio under a fume hood. Pour the Technovit 3040 solution onto the embedded sample's surface and attach a small wood block to the solution (Optional: a quick-drying glue can be used). Leave the samples until the Technovit 3040 solution becomes solid.

For preparing thin sections of the sample for microscopic observation, preheat the slide warmer to 42 °C. Place water droplets onto a MAS-coated slide glasses to the approximate size of the sections. Insert the wood block with sample into the sample holder of a Manual Rotary Microtome (Leica RM2235 or equivalent) and a TC-65 Tungsten blade to the blade holder of the microtome. Carefully slice the sample with manual rotation to 2–4 μm thickness. Hold the section with thin forceps and place each section onto the water droplet on the slide glass. Check the section under a light microscope. Place the slide glass onto the preheated slide warmer until the water dries up.

Double staining with Safranin-O and Fast Green. Preheat a hot plate (Corning 6795-420D hot plate stir or equivalent) to about 80 °C. Drop 0.1% Safranin solution on the slide containing the sample sections to cover them. Place the slide on the heat block until the Safranin solution is almost dried up. Briefly wash the slide glass with water. Set the slide(s) in a staining basket. Prepare the glass jars each filled with water, 0.5% picric acid, 95% ethanol, 95% ethanol with few drops of HCl, 100% ethanol, 0.1% Fast Green FCF (Fujifilm Wako Pure Chemical Industries, Ltd.) and 100% ethanol. Wash with water until no more red pigment comes off from the slide. Dip the slide glass in 0.5% picric acid in 95% ethanol for a few seconds. Next, dip the slide glass in 95% EtOH with ammonium solution for a few seconds. Dip the slide glass in 95% EtOH with a few drops of HCl. Wash with 100% ethanol. Stain with 0.1% Fast Green FCF for 30 s to 1 min (ideal duration may vary). Wash with 100% ethanol. Place the slide glasses on the slide glass holder and dry them in the air, and observe with a bright field microscope (Fig. 5a).

Simple staining with toluidine blue. The section on the slide glass can be stained with toluidine blue solution (Fig. 5b) instead of the double stainng listed above

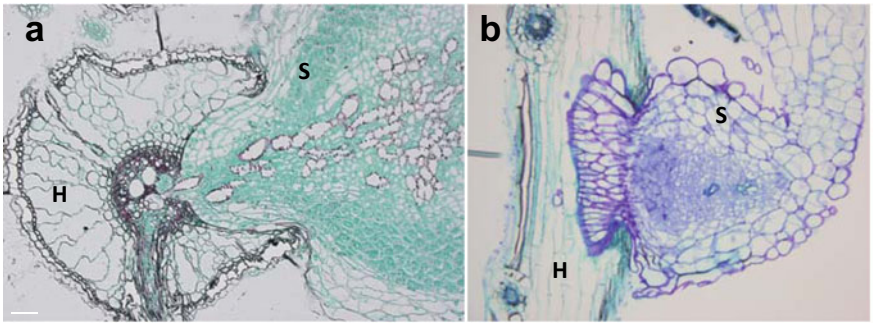

Fig. 5 Thin-sections *Striga hermonthica* infecting a rice root stained by different methods: **a** Safranin O and Fast Green double-stained cross-section of *Striga hermonthica* infecting a rice root. Scale bar, 50 μm. **b** toluidine blue-stained longitudinal section of *Striga hermonthica* infecting a rice root. S: *Striga*, H: rice

(Fig. 5a). Drop 0.01% toluidine blue solution (100 mg Toluidine Blue [Sigma-Aldrich] in 10 mL water) on the top of slide glass to cover the specimen. Wait for 2 min. Wash the slide glass by immersing in water. Observe with a bright field microscope.

Conclusion

The protocols herein described are useful for characterizing post-attachment *Striga* resistance in rice and sorghum. They may also be applied to maize with some modification (e.g., using larger plates to construct the rhizotrons). Although they can indicate pre-attachment resistance mechanisms, for instance by applying ungerminated preconditioned *Striga* seeds to host roots in the rhizotron, such observations are perhaps more suitable in the agar system described in Chapter "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum". The application of both co-culture methods can pinpoint the point at which resistance gained through mutagenesis counters *Striga* parasitism in cereal mutant lineages.

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Striga Germination Stimulant Analysis

Benjamin Thiombiano, Kristýna Floková, Aimee Walmsley, and Harro J. Bouwmeester

Abstract *Striga* is a flowering parasitic plant that can infect a broad range of crops including sorghum, maize and rice. The life cycle of *Striga* is intricately linked to its host, using host derived signals to initiate germination and attach to its host. Host susceptibility to *Striga* is thus directly correlated to the production and exudation of these signals. In this chapter we discuss the strigolactones, the most important class of *Striga* germination stimulants. We review the structural diversity of the strigolactones, their transport and biological relevance in plant and rhizosphere, and their importance for host specificity. In addition, we describe methods to analyse germination stimulant production by a *Striga* host: how to, reproducibly, collect the root exudate of a host, how to process these exudates, partially purify them and analyse them using analytical chemistry or a high-throughput image analysis-based germination bioassay.

Keywords Parasitic plant · Strigolactone analysis · Root exudate collection · *Striga* germination

Introduction

Selected *Striga* resistant cereal mutants derived from gamma irradiated stocks (Chapters "Screening for Resistance to *Striga* Hermonthica in Mutagenized Sorghum and Upland Rice in Burkina Faso"–"Phenotyping for Resistance to *Striga* Asiatica in Rice and Maize Mutant Populations in Madagascar") determined through laboratory assays to have lower *Striga* germination stimulant activity (Chapter "An Agar-based Method for Determining Mechanisms of *Striga* Resistance in Sorghum") than their unmutagenized progenitors may be further characterized by examining the strigolactones in their root exudates. This chapter provides further details of the parasitic

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association of *Striga* with its cereal hosts with particular emphasis on strigolactones, the germination stimulants in host root exudates. Protocols for collecting and chemical and biological characterization of root exudates are also described.

Striga *life cycle.* The life cycle of *Striga* commences with the germination of its seed, followed by its attachment to the host, marking the start of the parasitic phase. Before the seed can respond to germination stimuli, various metabolic processes occur in what is known as the "conditioning" phase. Full imbibition, which occurs within a day, must be followed by several days (optimally 10–14) of sustained moisture and warmth to become responsive to germination stimulants. If conditioned seed does not encounter a germination stimulant, it eventually returns to an unresponsive state of secondary dormancy (Matusova et al. 2004). With germination, the *Striga* radicle emerges from the seed coat and grows up to a centimeter. Once the radicle encounters a potential host root, its apex forms an haustorium that aids attachment and penetration of the epidermis, cortex and ultimately stele where xylem connection is established with the host (LeBlanc et al. 2012). A *Striga* shoot will only emerge with successful vascular connection. Germination without attachment and vascular contact with a suitable host will kill *Striga* since it cannot survive on its own seed reserves. An established parasite eventually emerges from the ground, flowers and sets up to 200,000 seeds/plant. This leads to the formation of vast seed banks, which pose a significant challenge in the management of parasitic weeds, as these seeds can remain dormant and viable in the soil for many years until they encounter a host.

Scientists had long ago observed that the root exudates of clover, maize, sorghum, and linseed contain compounds that induce the germination of parasitic plant seeds. (Saunders, 1933; Brown et al. 1949, 1951). The first witchweed (*Striga lutea* Lour.) germination stimulant was isolated from the root exudate of cotton (*Gossypium hirsutum* L.) and named strigol (Cook et al. 1966, 1972). Over 30 different molecules with similar properties to strigol, collectively referred to as strigolactones (SLs), have been discovered in the plant kingdom since then.

Germination stimulants. After its discovery in cotton, strigol was subsequently identified in the root exudates of *Striga* hosts such as maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), and proso millet (*Panicum miliaceum* L.) (Siame et al. 1993). Two additional germination stimulants were subsequently identified in the root exudates of sorghum, sorgolactone (Hauck et al. 1992), and from cowpea [*Vigna unguiculata* (Walp)], alectrol (which was later identified as orobanchyl acetate; Ueno et al. 2015; Xie et al. 2008). Both share basic chemical structure to strigol. In 1995, Butler coined the name strigolactones for these strigol-related compounds (Butler 1995). Some years later, orobanchol, a germination stimulant for the broomrape, *Orobanche minor* (Smith), was isolated from the root exudate of red clover (*Trifolium pratense* L.) (Yokota et al. 1998). This showed that SLs are used by both *Striga* and *Orobanche* spp. as germination cues to ensure germination in the presence of a host root. Subsequently, sorgomol (Fig. 1; Table 1) was identified in the root exudate of *Sorghum bicolor* and shown to induce germination in *Striga hermonthica* and *O. minor* (Xie et al. 2008). Fabacol, fabacyl acetate (germination stimulants of *O. minor*) and strigone (a highly active germination stimulant of *S. hermonthica*)

were isolated from the root exudates of pea (*Pisum sativum* L.) (Xie et al. 2009) and from the non-host, *Houttuynia cordata* (Kisugi et al. 2013), respectively.

The discovery of new, non-canonical SLs that do not possess the consistent ABCD-ring structure (as shown in Fig. 1) was a fascinating and unexpected development. One such example is the compound methyl carlactonoate (MeCLA), which has been found in *Arabidopsis* (van Ha et al. 2014; Seto and Yamaguchi 2014). Other examples are heliolactone, identified in the root exudate of sunflower (Ueno et al. 2014) and zealactone, identified in maize root exudate (Xie et al. 2017) that induces *S. hermonthica* germination (Charnikhova et al. 2017). Avenaol was identified in the root exudate of black oats (*Avena strigosa* Schreb.) and is a strong germination stimulant for *Phelipanche ramosa*, while it is virtually inactive in *S. hermonthica* and *O. minor* (Kim et al. 2014). To date, all examined plant species have been found to release a combination of multiple SLs. The SL profiles can vary among different species, and sometimes, even within different cultivars of the same species. Moreover, the quantities and proportions of SLs can fluctuate under varying growth conditions and developmental stages (Yoneyama et al. 2008).

Other roles of strigolactones. For many years, plant scientists were puzzled by the question of why plants exude SLs into the rhizosphere, thereby inviting parasitic weed attacks. In 2005, Akiyama et al. finally provided an answer to this quandary by demonstrating that SLs play a crucial role in establishing beneficial arbuscular mycorrhizal (AM) fungi symbiosis (Akiyama et al. 2005). Strigolactones stimulate hyphal branching in AM fungi, enabling them to extend towards and colonize the host root. This symbiotic relationship is a vital factor in the growth and survival of terrestrial plants, as demonstrated by its prevalence in approximately 80% of all plant species (Bonfante and Genre 2015; Gutjahr and Parniske 2013).

This mutualistic relationship plays a crucial role in providing the host plant with minerals that are absorbed by the fungal hyphae, effectively expanding the plant's root surface area and allowing it to exploit a much larger volume of soil. In exchange, the heterotrophic fungal partner receives reduced carbon compounds from the plant's photosynthesis to support its own growth (Gutjahr and Parniske 2013; Bonfante and Genre 2015). The benefits of AM symbiosis explain why plants, particularly under phosphorus deficiency, release SLs into the soil (Gutjahr 2014; Khosla and

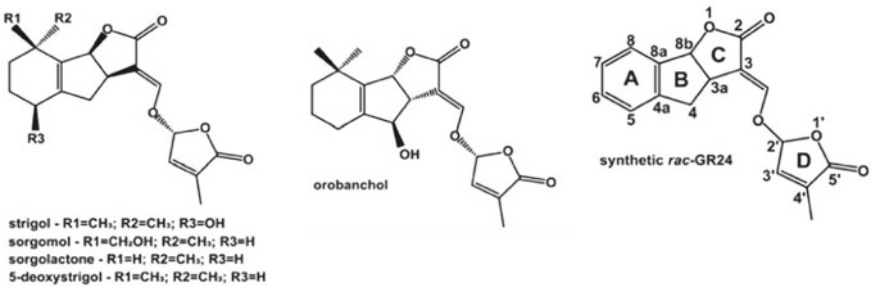

Fig. 1 Chemical structures of sorghum strigolactones and the synthetic strigolactone, GR24

Table 1 Optimized UPLC-MS/MS conditions for analysis of selected strigolactones

Authentic compound	Retention time (min)	MRM transitions			Cone voltage (V)	Collision energy (eV)	Internal standard[c]
		Parent ion $[M + H]^+$		Product ion			
Strigol	3.5	347.3	>	96.9[a]	20	20	GR24
			>	233[b]		18	
Orobanchol	4.59	347	>	97[a]	20	25	GR24
			>	205[b]		18	
Sorgomol	4.85	347.5	>	233[a]	22	18	GR24
			>	133[b]		25	
Sorgolactone	7.49	317.1	>	96.9[a]	20	20	$[^2H_6]$-5-DS
			>	133[b]		18	
5-deoxystrigol	8.01	331.1	>	96.9[a]	22	20	$[^2H_6]$-5-DS
			>	216[b]		18	
Internal standard							
GR24	5.13	299	>	96.9[a]	20	25	
			>	185[b]		25	
$[^2H_6]$-5-DS	7.95	337	>	96.9[a]	22	20	
			>	222[b]		18	

MS settings are following: capillary voltage 1.2 kV; ion source/desolvation temperature 120/550 °C; desolvation/cone gas flow 1000/150 L/h; collision gas flow 0.15 ml/min. Individual compounds were analyzed using characteristic quantifying (a) and confirming (b) MRM transitions. Internal standard (c) used for analyte quantification

Nelson 2016). It is plausible to assume that root parasitic weeds, which evolved much later than AM symbiosis, have co-opted the SL signal as a reliable indicator of host presence. This was achieved through the evolution of a highly sensitive SL detection system coupled with the induction of seed germination. It is worth noting that the function of SLs as a plant hormone was reported three years after their role in AM symbiosis was revealed (Gomez-Roldan et al. 2008; Umehara et al. 2008). The identification of SLs as a plant hormone regulating shoot branching and tillering was made possible by the availability of mutants with increased shoot branching and tillering from various plant species. Through genetic analysis and grafting studies, these mutants were classified into two groups: those deficient in the synthesis of a presumed mobile shoot branching-inhibitory signal and those affected in the perception of this signal. The biosynthetic mutants lack SLs, and application of the SL analog GR24 restores their branching and tillering phenotypes. This finding showed that SLs are the elusive shoot branching inhibitory signal. The supposed perception mutants, on the other hand, did not respond to GR24, which further supports the conclusion that SLs are the signal in question. (Gomez-Roldan et al. 2008; Umehara et al. 2008). Thus, SLs are a new plant hormone that plays an

important role in the adaptation of plants to nutrient deficiency, regulating senescence, root development, and stem secondary growth (Koltai 2011; Ruyter-Spira et al. 2013; Brewer et al. 2013; Al-Babili and Bouwmeester 2015; Waters et al. 2017; Jia et al. 2018). Moreover, SLs seem to play a role in the plant response to biotic and (other) abiotic stresses (van Ha et al. 2014; Torres-Vera et al. 2014; Wang et al. 2015; Decker et al. 2017).

Strigolactone biosynthesis. The discovery in 2008 that strigolactones act as the long-sought branching-inhibiting hormone (Gomez-Roldan et al. 2008; Umehara et al. 2008) facilitated the rapid elucidation of the core strigolactone biosynthesis pathway. This pathway involves a ß-carotene isomerase (DWARF27) and two carotenoid cleavage dioxygenases (CCD7 and CCD8), which produce carlactone, the precursor for all known strigolactones. However, biosynthesis of strigolactones diverges strongly after carlactone, leading to the production of different types of strigolactones, including orobanchol-type and non-canonical strigolactones that lack the classic ABC-ring structure of canonical strigolactones. Our understanding of the details of strigolactone biosynthesis after carlactone is limited, with only a few enzymatic steps identified in *Arabidopsis* towards non-canonical strigolactones and in rice towards orobanchol-type strigolactones (Bouwmeester et al. 2021). Notably, maize strigolactones with unique structures have been identified, but their biosynthesis remains unknown (Charnikhova et al. 2017).

Strigolactone transport. Grafting is a common technique used to study hormone and metabolite transport between plant shoots and roots. Mutants lacking strigolactones (SLs) exhibit a distinct high-branching phenotype. However, when these mutants were grafted onto wildtype rootstocks, the bushy phenotype was fully rescued, indicating that SLs are transported from roots to shoots (Kohlen et al. 2011). Conversely, grafting wildtype scions onto SL-deficient rootstocks did not result in increased branching, suggesting that SLs can also be synthesized in aerial tissues (Kohlen et al. 2011). Grafting studies have also shown that SL biosynthesis intermediates can be transported from roots to shoots. For example, grafting of MORE AXILLARY BRANCHING (MAX) mutants *max3* or *max4* onto *max1* rootstocks restored wildtype branching to the mutant shoots, suggesting that carlactone or its derivatives, which accumulate in *max1* rootstocks, are transported into the mutant shoots where they are further metabolized into SLs that inhibit branching (Booker et al. 2005).

Transport of SLs from roots to shoots has been suggested to occur through the xylem (Kohlen et al. 2011). However, labeled orobanchol and 4-deoxyorobanchol, that were applied to the roots, were detected in the shoot, but not in the xylem (Xie et al. 2015). Moreover, transport of the labeled SLs was slower than expected for xylem-mediated movement (Xie et al. 2015). In addition to transport from root to shoot, SLs are exuded into the rhizosphere by specific transporters, such as the Petunia PLEIOTROPIC DRUG RESISTANCE 1 (PhPDR1), an ATP-binding cassette (ABC) transporter (Kretzschmar et al. 2012). PhPDR1 is located in the plasma membrane of sub-epidermal cells in lateral roots, the hypodermal passage cells where AM fungi penetrate the roots. The activity of PhPDR1 may indicate the presence of a local maximum of SL, which could guide AM fungal hyphae towards the hypodermal

passage cells. PaPDR1, an ortholog of PhPDR1 found in *P. axillaris*, exhibits asymmetric localization in different root tissues, with its transporter localized at the apical membrane of root hypodermal cells in root tips, potentially facilitating the transport of SLs towards the shoot (Sasse et al. 2015). In hypodermal passage cells above the root tips, PaPDR1 is localized in the outer-lateral membrane, consistent with its postulated role in transporting SLs from roots into the rhizosphere.

SLs and host specificity. Many parasitic plant species display host specificity, often with a narrow host range, such as *O. cumana* that mostly only parasitizes sunflower. For other species, the host range is wider, such as *P. ramosa* that can infect several members of both Solanaceae and Brassicaceae (Gibot-Leclerc et al. 2016; Perronne et al. 2017). Within the broad host range *P. ramosa*, however, there are ecotypes that seem to exhibit a narrower host specificty. For example, the germination of *P. ramosa* seeds harvested from different hosts, is differentially stimulated by the exudates of these different hosts (Perronne et al. 2017). Also in *S. hermonthica*, although it can infect several cereals (maize, sorghum, millet, rice), we find ecotypes that better parasitize millet than sorghum and maize and vice-versa (Kim, 1994; Mohemed et al. 2018). Whether this specificity is based on the recognition of germination stimulants is an intriguing question. Indeed, the sunflower parasite, *O. cumana*, germinates in response to dehydrocostuslactone, a molecule present in the exudate of sunflower, but not to strigolactones (Auger et al. 2012). In *S. hermonthica*, strigolactone composition of the host root exudate seems to play a role in host specificity. For example, a sorghum ecotype of *S. hermonthica* germinates much less well with a millet exudate and vice-versa (Mohemed et al. 2018). In sorghum, maize and rice, *S. hermonthica* susceptibility seems to correlate with 5-deoxystrigol production (Jamil et al. 2012; Xie et al. 2015; Mohemed et al. 2018). In contrast, orobanchol seems to correlate with reduced susceptibility to *S. hermonthica* (Gobena et al. 2017; Mohemed et al. 2018).

Similar to auxin and other plant hormones, strigolactones also need a receptor to exert their effects on biological processes. The branched phenotype of mutants has been instrumental in identifying components involved in strigolactone perception and downstream signaling. This includes the discovery of two canonical α/β hydrolase fold proteins: the strigolactone receptor, D14, and the homologous receptor, D14like1/KAI2/HTL (abbreviated as HTL) (Waters et al. 2017). D14 is a receptor with hydrolase activity and the strigolactone ligand is hydrolyzed upon binding (Seto et al. 2019). Although the crystallographic studies indicate that the D-ring is essential for the interaction between D14 and MAX2, it remains uncertain whether hydrolysis is a prerequisite for signaling or merely a result (Yao et al. 2016; Seto et al. 2019). When a strigolactone or the D-ring binds to D14, it causes a conformational change that allows D14 to recruit the F-box protein MAX2/D3. MAX2/D3 then targets repressor proteins such as DWARF53 and SMXLs for ubiquitination, leading to their proteasomal degradation. As a result, gene expression is induced and plant development is altered, including the inhibition of bud outgrowth (Al-Babili and Bouwmeester 2015). While D14 has been confirmed as the receptor responsible for regulating plant development through strigolactones, several impressive studies have

demonstrated that HTL has evolved new ligand binding specificity in root parasitic broomrapes and witchweeds through duplication and adaptation (Conn et al. 2015; Toh et al. 2015). This facilitated germination of the parasites upon perception of strigolactones secreted by their host. Intriguingly, the function and ligand of HTL in non-parasitic plants is unknown (Waters et al. 2017). With the strigolactone receptors in hand we can now screen for ligands. Uraguchi et al. (2018), for example, used a *S. hermonthica*, HTL to find new synthetic suicidal germination stimulants (Uraguchi et al. 2018).

Testing individual SLs for their ability to induce parasitic plant seed germination has confirmed that seeds of different species differentially respond to specific SLs (Wang and Bouwmeester 2018). For example, germination of *O. minor* is induced with about 200-fold lower *ent*-2'-*epi*-orobanchol concentration than *S. hermonthica* (Ueno et al. 2011). Similarly, in the same concentration, 5-deoxystrigol only induced germination in *S. hermonthica* but not in of *S. gesnerioides* (Ueno et al. 2011).

Role of SLs in other belowground interactions of the host. Plants utilize multiple adaptation strategies when exposed to stress such as phosphate or nitrogen deficiency. The most crucial ones include altering the architecture of roots and shoots, establishing beneficial interactions with microorganisms, and modifying the pH of the rhizosphere (Bouwmeester et al. 2007; Péret et al. 2011; Auger et al. 2012; Kumar et al. 2015). All of these mechanisms aim to enhance the availability of nutrients for plants. It is interesting to note that when plants are grown in media deficient in nitrate, and particularly phosphate, there is a stimulated increase in the production of SLs (López-Ráez et al. 2008; Yoneyama et al. 2012; Marzec et al. 2013; Torres-Vera et al. 2014). There are various indications that the up-regulation of SL production is involved in the plant adaptation to low-nutrient conditions. For instance, in the absence of phosphate, plants tend to prioritize the production of lateral roots (Péret et al. 2011), which increases the surface area in contact with the soil. This adaptation is facilitated by the important role of auxin in initiating lateral root primordia and promoting the emergence of lateral roots (Chiou and Lin 2011; Sun et al. 2014), but SLs also play an important role in the adaptation of root architecture to phosphate deficiency (Ruyter-Spira et al. 2011; Sun et al. 2014; Kumar et al. 2015) and, in cross talk with auxin, SLs induce an increase in lateral root density (Ruyter-Spira et al. 2011). The alteration in root architecture that occurs in response to low-nutrient conditions may also impact the susceptibility of host plants to parasitic infections. This modification increases the likelihood of host roots encountering parasitic plant seeds. Furthermore, the increased production and release of SLs in response to nutrient deficiency not only promotes the colonization of plant roots by beneficial microorganisms but also stimulates the germination of parasitic plant seeds, thereby increasing the risk of infection (Jamil et al. 2012, 2014).

In addition to the interaction with AM fungi (Akiyama et al. 2005; Besserer et al. 2006), SLs were also shown to regulate nodulation. The SL deficient increased branching (*ramosus*) pea *rms1* mutant displayed a reduced nodule number (Foo et al. 2013). Also in soybean, nodulation was decreased in the SL deficient *GmMAX3b* mutant line while overexpression resulted in an increased nodule number (Haq et al.

2017). The precise mechanism behind this impact of SLs on nodulation is not fully understood, including whether it is due to their signaling function or hormonal effect.

In addition to SLs, plants also exude a wide range of other chemicals into the rhizosphere. These molecules provide carbon for microorganisms and serve as signaling molecules that play a critical role in recruiting and selecting specific microorganisms. Among the most extensively studied examples are phenylpropanoids, which are involved in both symbiotic and pathogenic interactions in the rhizosphere (Abdel-Lateif et al. 2012; Liu and Murray 2016) and allelopathy (Bais et al. 2006).

Recent work on sorghum showed that the *Striga* resistant genotype SRN39, which exudes mostly the SL orobanchol (Gobena et al. 2017) recruited a different microbiome than other genotypes that mostly exude 5-deoxystrigol (Schlemper et al. 2017). Also in rice a significant correlation was detected between orobanchol and a number of micro-organisms, including AM fungi and *Burkholderia–Caballeronia–Paraburkholderia* and *Acidobacteria* that potentially solubilize phosphate (Kim et al. 2022). Observations suggest that these changes at the microbiome level can mitigate infection of the host by parasitic plants. From a *Striga* suppressive Kenyan soil, bacteria were isolated that induced up to 45% of decay in *Striga* seeds (Neondo et al. 2017). Other mechanisms by which soil micro-organisms could suppress parasitic plants include the production of germination inhibiting factors, inhibitors of radicle growth and haustorium formation, strengthening the vigor of the host plant by activating plant defense mechanisms or competitive utilization of signaling molecules inducing parasitic plant seed germination.

SLs and parasitic weed management. Controlling parasitic Orobanchaceae weeds poses several challenges. On the one hand, their parasitic nature makes it difficult to intervene without harming the host plant, due to the physical and physiological connection between the two. On the other hand, the properties of their seeds, such as their small size, fecundity, and easy dispersal, make it challenging to prevent the spread of the weed. These factors lead to a rapid increase in the soil seed bank. Therefore, preventing the distribution of seeds and containing infested areas should be a significant focus of any parasitic weed management strategy, in addition to direct control measures against the parasites (Rubiales et al. 2009; Rubiales and Fernández-Aparicio 2012). In this Chapter, we will not review all possible control and management strategies of parasitic weeds, but focus on methods—that are already used or can potentially be developed—that are based on the importance of SLs in the lifecycle of these parasites. Indeed, there are several strategies of weed management focusing on the SLs, trying to avoid the stimulation of germination, or conversely to favor it, in the absence of a host. These practices are briefly considered in the next sections.

Host resistance through low strigolactone exudation. As discussed above SLs are the main germination stimulants for root parasitic plants. In studies that evaluated the induction of parasitic plant seed germination by exudates from different genotypes and cultivars of several crop species, a positive correlation was demonstrated between the SL concentration in the root exudate and the germination rate (Jamil et al. 2011a, b; Fernández-Aparicio et al. 2014; Yoneyama et al. 2015; Mohemed et al. 2018).

An approach for the management of parasitic plants in agricultural crops could thus be to reduce germination of the parasitic plant seeds by reducing the exudation of the germination stimulants. Several studies explored natural variation in germination stimulant production, for example in the New Rice for Africa (NERICA) rice cultivars. This work showed that several NERICA cultivars (1, 2, 5, 10 and 17) displayed post-germination resistance to *S. hermonthica* and *S. asiatica* unlike NERICA 7, 8 and 11, which were susceptible (Rodenburg et al. 2015). In parallel, variation in SL production in the NERICA genotypes was demonstrated, resulting in differences in *Striga* germination induction (Jamil et al. 2011b). The combination, by breeding, of germination-related resistance with post-germination resistance could result in better durable *Striga* resistance (Cissoko et al. 2011; Jamil et al. 2011b).

A pea genotype with reduced SL exudation displayed partial field resistance to *O. crenata* infection (Pavan et al. 2016). Further in sorghum, the *Striga* resistant genotype SRN39 produced much less 5-deoxystrigol than the susceptible Tabat (Yoneyama et al. 2010). Later, SRN39 was shown to produce more orobanchol, instead of 5-deoxystrigol, due to a tentative modification in the SL biosynthetic pathway (Gobena et al. 2017). This mechanism was also observed in a number of other *Striga* resistant sorghum genotypes (Mohemed et al. 2018). Interestingly, the differences in SL composition in maize and sorghum did not affect the level of AM colonization (Gobena et al. 2017).

Besides exploiting natural variation, biotechnological approaches aiming to generate low SL exuding plants could be a strategy to reduce infestation by parasitic plants (Torres-Vera et al. 2014). Indeed, it was demonstrated in tomato that by knocking down SL biosynthesis through genetic modification, the mutant was more resistant to *O. ramosa* infection (Kohlen et al. 2012). A reduction in SL production to obtain parasitic weed resistance was also achieved unintentionally. The broomrape resistant tomato mutant Sl-ORT1 developed by Dor and coworkers was later found out to be SL-deficient and the resistance thus associated to the low amount of exuded SLs (Dor et al. 2010). Breeding, through conventional or biotechnological approaches, for a reduction in exuded SLs potentially also has negative consequences given their importance for the control of shoot and root architecture and the acquisition of nutrients through AM fungi (López-Ráez et al. 2008). This could possibly be prevented by approaches that reduce transport of SLs into the rhizosphere, which is facilitated by an ABC transporter, PDR1 (Borghi et al. 2015). However, under certain abiotic stress conditions, this could still negatively affect the adaptive capacity of plants by hampering mycorrhization. Particularly the example of sorghum shows that solutions in which the composition rather than the level of the SLs is changed may be the best solution (Gobena et al. 2017). Nevertheless, several examples show that a reduction in SL production results in an acceptable level of resistance without large consequences for the plant phenotype (Jamil et al. 2011a, b; Pavan et al. 2016). In order to prevent that this partial germination-based resistance is overcome by parasitic plant populations, a combination of pre-and post-attachment resistance mechanisms is necessary.

In this chapter we describe how to analyse the level of germination stimulant produced by a *Striga* host, with an emphasis on the SLs as the most important class

of *Striga* germination stimulants. With the methods described here it is possible to screen collections of germplasm and/or mutants suspected to induce lower *Striga* germination, to find genotypes that display (partial) *Striga* resistance through a lower (or no) induction of *Striga* germination. We describe how to reproducibly collect the root exudate of hosts, how to process these exudates, partially purify them and analyse them using a *Striga* germination bioassay, for which we will also describe a method to analyse the results through high-throughput image analysis, or using advanced Multiple Reaction Monitoring—Liquid Chromatography Coupled to Mass Spectrometry (MRM-LC-MS).

Protocols

Root exudate collection. One of the of plant adaptive characteristic features is their capacity to constantly communicate with their surrounding environment through metabolites. These chemical compounds are widely diverse and involved in a broad range of responses: attraction of beneficial or harmful micro-organisms, iron chelation, rhizosphere pH modulation, nutrient uptake, etc. It has been shown that to face a specific biotic or abiotic stress plants modulate the composition of their root exudates to attract the right beneficial micro-organisms. Due to coevolution, harmful organisms are often able to hijack these signals. A well-studied dual effect compounds example are the strigolactones (SLs) that the plant uses to attract arbuscular mycorrhizal fungi and that also induce parasitic plant seed germination. In their attempt to mitigate phosphate deficiency through mycorrhization, SL levels in roots and root exudates increase under phosphate stress. Therefore, characterizing the SL profiles of *Striga* hosts is often done under phosphate starvation. In addition to manipulating nutrition, temperature and light to optimize SL exudation, other aspects such as SL stability need to be considered while collecting root exudates. The following steps have been successfully used in our lab to collect root exudates from cereal *Striga* hosts like maize, millet, rice and sorghum.

Place the desired number of deglumed seeds in a 15 ml Falcon tube. Add 10 mL of 4% sodium hypochlorite with 0.2% Tween-20 and shake the tube for 45 min on a rotating mixer. After removing the bleach, soak the seeds for 30 s in 10 mL of 70% ethanol and wash with 10 mL of sterile MilliQ water (repeat this step three times). Wash the seeds an additional four times with 10 mL of sterile MilliQ water.

Moisten autoclaved 90 mm Whatman® glass microfiber filters (GF/A Grade/ qualitative filter paper) with 3 mL sterile MilliQ water in a sterile 90 mm petri dish, then place 10–15 seeds on each filter paper. Seal the plates with parafilm and wrap them with aluminum foil, then incubate them for two days at 30 °C. Select the best seedlings and transfer them to 50 mL Falcon tubes filled with 45 mL of washed sand. Sand can be prewashed by placing a bulk quantity in a sieve and placing under running water. Move the planted tubes to a greenhouse or growth chamber set at 28 °C/25 °C, 70% relative humidity, 16 h photoperiod, 450 μmol. photons m^{-2} s^{-1}.

This light intensity is ideal but a range of 200–450 μmol. photons m^{-2} s^{-1} could be used. Select the better growing plants after two days. Water the plants every two days with 5 mL of modified half strength Hoagland's solution, containing 10% of the normal amount of phosphate. The use of sand for plant growth gives a relatively clean exudate and facilitates the low nutrient treatment. However, it has the disadvantage of drying very quickly. Daily watering is therefore very important for the first three days after the transfer of the seedlings. Grow the plants under these conditions for two weeks.

Elute the exudates with 30 mL of 5% ethanol–water and collect 20 mL. The analyte enrichment can be improved by addition of a suitable organic modifier at 5% to the root exudate collection solvent; also see below (Floková et al. 2020). Keep the exudates on ice for the whole exudate collection process. During the root exudate collection, the stability of SLs can be an issue. Keep the exudates therefore refrigerated until they are combined with the solid-phase extraction (SPE) sorbent. Centrifuge the exudates (4 °C, 3000 rpm for 20 min) and transfer supernatant to a second Falcon tube.

Processing the exudates, preserving and storing samples. The process of sample extraction and further purification significantly influences the overall sensitivity of final UHPLC-MS/MS analysis. The universal solid phase polymer-based sorbent for neutral compounds Oasis HLB® (Waters), combining both hydrophilic and lipophilic retention characteristics, was successfully applied in pre-concentration of broad spectrum of strigolactones (Floková et al. 2020). The additional selective purification step using polar interactions with silica sorbent eliminates the co-extraction of lipophilic contaminants, greatly improves the sample purity and decreases detection limits of mass analysis on the sample background. The complete sample preparation procedure is shown in Fig. 2.

Filter ~ 100 mL of collected exudates using a Büchner vacuum funnel[1] and transfer 20 mL of filtered exudates to a 50 mL Falcon tube. Add the mixture of internal standards (authentic or stable deuterium-labelled counterparts of strigolactones) to the sample and vortex.[2]

Before sample concentration with SPE column, activate the sorbent of Oasis HLB® with 100% acetonitrile (3 mL) and subsequently equilibrate with 3 mL of 5% ethanol/water (v/v). Solvents and sample extracts are passed through the SPE cartridge Oasis HLB® at a consistent flow rate 1–2 drops per second.

Load the extract of root exudates onto conditioned SPE sorbent (~ 50 mL). Discard the flow-through fraction. Wash sorbent with deionized water (3 mL) to remove

[1] The complete set up for pressure assisted filtration consists of a Büchner vacuum filter funnel (100 mL volume), side-arm flask (250 mL volume), adapters, silicone pump tubing, portable diaphragm vacuum pump (max. vacuum 160 mbar), grade 1 filter paper with pore size 11 μm.

[2] Internal standard stock solutions are generally prepared beforehand by dissolving each standard in 50% acetonitrile/water (v/v) to final concentration 10^{-5} M, and stored at − 20 °C. Working solution of internal standards is a mixture of stock solutions, diluted to final concentration 5×10^{-7} M. The mixture of internal standards used in this example was GR24 and [$^2H_6$]-5-deoxystrigol prepared in 50% acetonitrile/water (v/v) mixed to final concentration 5×10^{-7} M (5 pmol of each compound in 10μL of solution).

Fig. 2 Analysis of *Striga* germination stimulants. Root exudate collection and concentration for strigolactones measurement using UPLC-MRM-MS/MS (left panel) and image analysis pipeline for analysis of *Striga* germination rate (right panel)

salts and unretained compounds. Discard the flow-through fraction. Place 4 mL borosilicate glass vials in the rack of a solid-phase extraction (SPE) glass block vacuum manifold (e.g., 24-port manifold lid) and perform the elution with 80% acetone (v/v, 3 mL). Evaporate samples to dryness using vacuum sample concentrator. Keep dry samples at − 20 °C until the following purification step.

To purify the sample, dissolve dry sample in 150 μL of 100% ethyl acetate and vortex. Fill the reconstituted sample up to 3 mL with 100% hexane (2.85 mL). Before sample purification with SPE column, activate the sorbent of Strata® SI-1 Silica with 100% ethyl acetate (3 mL) and subsequently equilibrate with 100% hexane (3 mL). Solvents and sample extracts are passed through the SPE cartridge Strata® SI-1 Silica at a consistent flow rate 1–2 drops per second. Avoid drying out the silica-based SPE sorbent bed and loss of column capacity. Load dissolved sample onto conditioned

SPE sorbent (3 mL). Discard the flow-through fraction. Wash the sorbent with 100% hexane (3 mL). Discard the flow-through fraction. Place 4 mL borosilicate glass vials in the rack of vacuum manifold and perform the elution with 80% ethyl acetate/hexane (v/v, 3 mL). Evaporate samples to dryness using vacuum sample concentrator. Keep dry samples at − 20 °C until the final LC–MS analysis.

Germination stimulant analysis using UPLC-ESI–MS/MS: strigolactones. Ultra-high performance liquid chromatography (UHPLC) coupled to fast scanning tandem mass spectrometry (MS/MS) is routinely applied for simultaneous profiling and quantitation of strigolactones, isolated from different sample matrices (Boutet-Mercey et al. 2018; Floková et al. 2020; Rial et al. 2019, p. 20; Sun et al. 2014). After analyte enrichment and additional purification using SPE-based sorbents, special attention has to be paid to suitable chromatography system and mobile phase composition. The sufficient retention of strigolactones is achieved on reversed-phase ethylene-bridged hybrid polymer-based columns (BEH) packed with sub-2-micron particles, improving both separation efficiency and speed of analysis (Fig. 3). The presence of eluent additive formic acid in dual-component mobile phase (water/acetonitrile) facilitates the chromatographic resolution between strigolactone stereoisomers with the same MS-fragmentation pattern, such as 5-deoxystrigol and 4-deoxyorobanchol (Floková et al. 2020). However, the main reason of mobile phase acidification is to support protonation of the strigolactone molecules $[M + H]^+$ in the electrospray (ESI) source, operating in positive mode, and thus improve analyte extraction by the mass analyzer. Compounds are analysed in multiple reaction monitoring mode (MRM) using two specific precursor-to-product ion transitions, listed in Table 1.

To analyse SLs, reconstitute the dried sample with 100 μL of 25% acetonitrile/water (v/v) and vortex. Transfer the dissolved sample onto the membrane of a 750 μL micro-centrifugal nylon filter with pore size 0.2 μm and spin in a pre-cooled (4 °C)

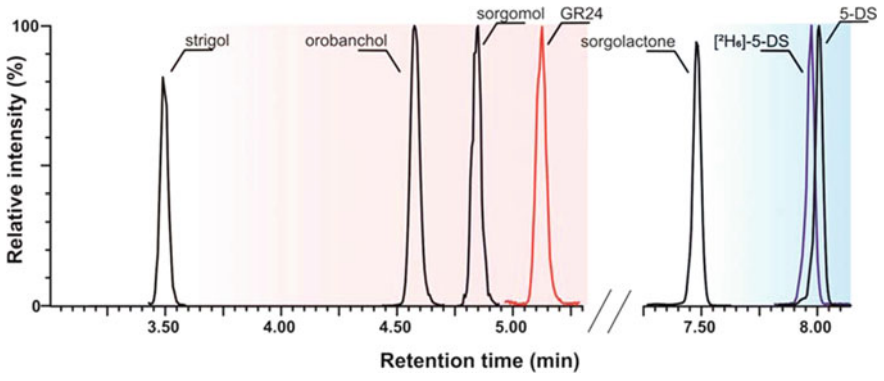

Fig. 3 The chromatographic separation of five authentic strigolactones coupled to internal standards with similar retention time: strigol, orobanchol and sorgomol to GR24 (red peak line); sorgolactone and 5-deoxystrigol (5-DS) to [$^2H_6$]-5-deoxystrigol ([$^2H_6$]-5-DS, blue peak line). Separation is performed on Acquity UPLC™ BEH C18 column

centrifuge at 10,000 g for one minute. Transfer the eluent to a 250 μL glass insert in a 1.5 mL LC/MS autosampler vial. Place the vials in the autosampler of the UPLC System,[3] maintained at 10 °C. Inject 5 μL of the sample onto an analytical Acquity UPLC™ BEH C18 column (2.1 × 100 mm, 1.7 μm particle size). Separate analytes with a 12 min binary gradient elution, consisting of 15 mM formic acid in both water (eluent A) and acetonitrile (eluent B), at flow rate 0.45 mL/min and constant temperature of 45 °C. Set the elution profile as follows: 0–0.4 min initial isocratic elution at 15% B; linear gradient increase to 27, 40 and 65% B in 0.65, 5 and 8 min, respectively; isocratic elution at 65% until 8.7 min. Finally wash the column with 95% B for 1.6 min and equilibrate back to initial conditions (15% B) for 1.7 min. The retention time of individual compounds is listed in Table 1.

Introduce the eluate in-line into the electrospray ion source (ESI) of the tandem mass analyzer, extract positively charged parent mass $[M + H]^+$ and determine strigolactones in multiple reaction monitoring mode (MRM) using characteristic quantifying and confirming fragment of individual analytes. Settings of the mass spectrometer are: capillary voltage 1.2 kV; ion source/desolvation temperature 120/550 °C; desolvation/cone gas flow 1000/150 L/h; collision gas flow 0.15 mL/min. Optimized conditions for each compound, such as cone voltage, collision energy and dwell time, are listed in Table 1.

To calibrate, inject a standard mixture with varying concentration levels of authentic analytes and quantify strigolactones using internal standardization method. A calibration curve is constructed by serial dilutions of authentic standards in the range from 5×10^{-7} to 2.5×10^{-10} M, containing a fixed amount of internal standard. Make a plot of known concentration of authentic standards to the ratio of peak areas for analyte vs. peak area for internal standard (response, Table 1). The internal standardization is a quantitative method of analysis, used to compensate for analyte losses during the sample preparation procedure and loss of signal during the ESI ionization process. The analyte is quantified based on solid signal of the appropriate reference standard, added to sample at the known concentration. Individual strigolactones are coupled to synthetic structural analogues with similar chromatographic behavior, listed in Table 1.

Striga *germination bioassay*. Seeds of parasitic Orobanchaceae will only germinate if they perceive the presence of SLs, which in the field means they are within the host rhizosphere and thus after germination they have a better chance to rapidly attach to the host root. A simple bioassay based with variation on that developed by Mangnus et al. (1992) for studying SLs has been used extensively since the discovery of their role as germination stimulants. It is based on in vitro reproduction of the steps necessary for parasitic seeds to germinate. The conditioning requirement, as described previously, is met by keeping seeds in warm moist conditions (e.g., wet filter paper in a petri dish at 25 °C) for several days. Conditioned seeds are then

[3] The chromatographic system used is a Waters Acquity UPLC™ I-Class System (Waters, Milford, MA, USA) equipped with Binary solvent manager and Sample manager coupled to Xevo® TQ-XS tandem quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray (ESI) ionization interface with MassLynx™ software, version 4.2 (Waters) to control instrument, acquire and process MS data.

exposed to the stimulant at proper concentration (usually at ppm or ppb levels) to induce germination which generally occurs within a day or two. Several observations can then be performed, e.g., percentage of germination, shape and length of the germination tubes and seed viability. More recently, high-throughput germination bioassays have been developed based on a standardized 96-well plate test coupled with spectrophotometric reading of tetrazolium salt (MTT) reduction (Pouvreau et al. 2013). These bioassays can be useful for guiding the purification steps for the identification of novel stimulants, testing dose–response effectiveness of SLs or their derivatives and analogues, evaluating SL selectivity/specificity in parasitic species/strains, assaying germination inhibitors or studying the physiology of the first stages of the parasitism. The complete *Striga* germination bioassay procedure is shown in Fig. 2.

To surface sterilize and condition *S. hermonthica* seeds, place the desired amount in a 15 mL Falcon tube. Add 10 mL of 2% sodium hypochlorite with 0.02% Tween-20 and invert the tube for five minutes. After removing the bleach, wash with 10 mL sterile MilliQ water 4–8 times until the water becomes clear. Moisten autoclaved 90 mm glass fiber filter papers (GF/A) set in a sterile petri dish with 3 mL sterile MilliQ water, then spread up to 100 mg of *Striga* seeds on each filter paper. Seal the plates with parafilm and wrap them with aluminum foil, then incubate them at 30 °C for 5–12 days.

To test germination, prepare 0.1% agarose containing 50–100 times diluted exudate or containing another compound of interest (e.g., 1 ppm GR24) in 2 mL Eppendorf tubes. It is not necessary to use GR24 as positive control. One may use any strigolactone that is available to them, and can choose a different concentration as well. Add preconditioned *Striga* seeds to the solution, and shake the tubes. Transfer 650 μL of *Striga* in solution into each well of a 12-well plate. Seal the plates with parafilm and wrap them with aluminum foil, then incubate them at 30 °C for two days.

Take a picture of each well using a binocular microscope with 10× magnification, or using a camera with a macro lens with 10× magnification at a set distance to have exactly one well in each picture. Regardless of with what instrument the picture is taken, a light background is necessary. A binocular microscope is the most obvious choice, but other options may be possible. Also, a relatively specific white balance (causing a bluish tint) is needed to obtain the contrast necessary for the automated image analysis.

Striga *germination assay automated image analysis*. Germination counts from the images can be automated using *ImageJ* (v1.53, available for free download from the United States National Institutes of Health at https://imagej.nih.gov/ij/) and *Rstudio* (v.1.1.453, a free, open source integrated development environment for R available for download at https://www.rstudio.com/products/rstudio/download/) software. A computer to run the software should have a minimum of 64-bit operating system, Windows 7 or greater, Intel® Core™ i5 and 8 GB RAM. Save the pictures to be analyzed in one folder. Save the pictures as TIFF files, or use another format that is supported by *ImageJ*. Open *ImageJ*, and go to Analyze > Set Measurements. Select Area, Shape descriptors, Perimeter, Feret's diameter, and Display label. Next, go to

Process > Batch > Macro. Select the input folder containing the photos to be analyzed. Select the output folder, which is where altered/analyzed pictures will be created (if the input and output folder are the same, the original picture will be written over). Batch process the folder with the pictures using the supplied *ImageJ* macro. Save the result table as an Excel file. If one is only interested in the germination rates, and not in the rate of haustorium formation, the macro can be slightly adjusted so that the results table will not be formed (by removing 'display' when using 'analyze particles' to count the number of germinated *Striga* seed. Open the result file in *Rstudio*, and add necessary columns to the data (e.g. treatment and concentration). Run the provided R script to obtain the results in tables, graphs, and statistical tests.

ImageJ Macro

```
*Altering file names*
a=getTitle();
dotIndex=indexOf(a, ".");
b=substring(a, 0, dotIndex)
rename(a+"_total_seeds");
run("Duplicate…", " ");
rename(a+"_Germinated");
selectWindow(a +"_total_seeds")

*Thresholding the Hue slice (these are settings used for a specific combination of camera settings, and may require adjustments for other users)*
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Saturation");
close();
selectWindow("Brightness");
close();
selectWindow("Hue");
setThreshold(0, 120);
run("Make Binary");

*Selecting the area of interest within the photo (needs to be consistent within an experiment to give accurate results, and may need readjusting between experiments)*
makeOval(100, 100, 2750, 2750);
run("Clear Outside");
run("Despeckle");
rename(b+"_Seeds");

*Set the scale of the picture in order to convert pixels into length in mm and count the number of seeds*
run("Set Scale…", "distance=2822 known=21.6 pixel=1 unit=mm");
```

```
run("Analyze Particles…", "size=0.012–0.2 circularity=0.2–1 display include add");
close();
```

Selecting the area of interest within the Blue slice, which should be the same as for the Hue slice (needs to be consistent within an experiment to give accurate results, and may need readjusting between experiments)

```
run("RGB Stack");
run("Convert Stack to Images");
selectWindow("Red");
close();
selectWindow("Green");
close();
selectWindow("Blue");
makeOval(100, 100, 2750, 2750);
run("Clear Outside");
```

Thresholding the Blue slice (these are settings used for a specific combination of camera settings, and may require adjustments for other users)

```
setThreshold(0, 100);
run("Convert to Mask");
run("Make Binary");
run("Despeckle");
rename(b+"_Germinated");
```

Set the scale of the picture in order to convert pixels into length in mm (same as for hue slice) and count the number of germinated seeds

```
run("Set Scale…", "distance=2822 known=21.6 pixel=1 unit=mm");
run("Analyze Particles…", "size=0.052–0.5 circularity=0.05–1 display include add");
close();
```

R Script

General notes about naming before starting analysis

- This analysis only works when pic_info file contains 2 columns (picture name/ number & treatment): Beware of how you name the treatments!

-> for more indepth comparisons, you may want to add more columns to your pic_info file (e.g. concentration, compound, sample type, rep, …), but you'll need to adjust the script accordingly

Set working directory & open imageJ results file (original csv file), and pic info file (excel converted to txt file)

```
setwd("C:/Users ")
```

```
Results <- read.csv("Results.csv", sep=";")
Pic_info <- read.table("Pic_info.txt", header=T, colClasses='character')

*Open the needed libraries*
library(tidyr)
library(reshape2)
library(ggplot2)
library(car)

*Merge ImageJ output with Pic_info file*
Results <- separate(data=Results, col=Label, into=c("del", "Image", "Type"), sep="_")
Results <- merge(Results, Pic_info, by="Image", all.y=TRUE)

*OPTION A: Remove extra columns (if second-forth column are the separated label)*
Results <- Results[c(1,15,3,4,5,7,11,12,14)]

*OPTION B: Remove extra columns (if second column is nameless with numbers, and the third-fifth column are the separated label)*
Results <- Results[c(1,16,4,5,6,8,12,13,15)]

*Create subset tables for seeds and for germinated counts*
Seed_Results <- subset(Results, Type=="Seeds")
Seed_Results <- Seed_Results[c(1,2,4)]
Seed_Results["Seed_Count"] <- as.integer(ifelse(Seed_Results$Area < 0.1, "1", ifelse(0.1 <=Seed_Results$Area & Seed_Results$Area < 0.15, "2", ifelse(0.15 <=Seed_Results$Area & Seed_Results$Area < 0.2, "3", ifelse(Seed_Results$Area > =0.2, "4", NA)))))
Germinated_Results <- subset(Results, Type=="Germinated")
Germinated_Results["FM"] <- Germinated_Results$Feret / Germinated_Results$MinFeret
Germinated_Results["PFS"] <- Germinated_Results$Perim. / (Germinated_Results$Feret / Germinated_Results$Solidity)
Germinated_Results["PAR"] <- Germinated_Results$Perim. * Germinated_Results$AR
Germinated_Results["PFS.FM"] <- Germinated_Results$PFS/Germinated_Results$FM
Germinated_Results["Haust_Count"] <- as.integer(Germinated_Results$Feret < 0.6 & Germinated_Results$FM < 1.8 & Germinated_Results$PFS > =2.3 | Germinated_Results$Feret > =0.6 & Germinated_Results$FM < 2 & Germinated_Results$PAR < 4.8 & Germinated_Results$PFS > 2)
```

```
Germinated_Results["Int_Count"] <- as.integer(Germinated_Results$Feret > =
0.6 & Germinated_Results$PFS > =2 & Germinated_Results$FM < 2 & Germi-
nated_Results$PAR > =4.8 | Germinated_Results$Feret > =0.6 & Germi-
nated_Results$PFS > =2 & Germinated_Results$FM > 2 & Germinated_
Results$PFS.FM > =0.9 & Germinated_Results$PAR > =4.8)
Germinated_Results["Rad_Count"] <- as.integer(Germinated_Results$Feret <
0.6 & Germinated_Results$FM > =1.8 & Germinated_Results$PFS > =
2.3 | Germinated_Results$Feret < 0.6 & Germinated_Results$PFS < 2.3 |
Germinated_Results$Feret > =0.6 & Germinated_Results$PFS < 2 | Germi-
nated_Results$Feret > =0.6 & Germinated_Results$PFS > =2 & Germi-
nated_Results$FM > =2 & Germinated_Results$PFS.FM < 0.9 | Germi-
nated_Results$Feret > =0.6 & Germinated_Results$PFS > =2 & Germinated_
Results$FM > =2 & Germinated_Results$PFS.FM > =0.9 & Germinated_
Results$PAR < 4.8)


*Summarize all results per picture*
Summary_per_Pic <- aggregate(Seed_Results[, 4], by=list(Treatment=Seed_
Results$Treatment, Image=Seed_Results$Image), sum)
Germinated_per_Pic <- aggregate(Germinated_Results[, c(14:16)],
by=list(Treatment=Germinated_Results$Treatment, Image=Germinated_
Results$Image), sum)
Summary_per_Pic <- merge(Summary_per_Pic, Germinated_per_Pic,
all.x=TRUE)
Summary_per_Pic["Germinated_Count"] <- Summary_per_Pic$Haust_
Count+Summary_per_Pic$Int_Count+Summary_per_Pic$Rad_Count
Summary_per_Pic[is.na(Summary_per_Pic)] <- 0
names(Summary_per_Pic) <- c("Treatment", "Image", "Seed.Count", "Haust_
Count", "Int_Count", "Rad_Count", "Germinated.Count")
Summary_per_Pic["Germination.rate"] <- Summary_per_
Pic$Germinated.Count/Summary_per_Pic$Seed.Count*100
Summary_per_Pic["Haust.Percentage"] <- Summary_per_Pic$Haust_Count/
Summary_per_Pic$Germinated.Count*100
Summary_per_Pic["Int.Percentage"] <- Summary_per_Pic$Int_Count/
Summary_per_Pic$Germinated.Count*100
Summary_per_Pic["Rad.Percentage"] <- Summary_per_Pic$Rad_Count/
Summary_per_Pic$Germinated.Count*100
Summary_per_Pic <- Summary_per_Pic[c(2,1,3,7,8,4,5,6,9,10,11)]
Germination_per_Treatment <- do.call(data.frame, aggregate(Summary_per_
Pic[, 5], by=list(Summary_per_Pic$Treatment), function(Summary_per_Pic)
c(mean=mean(Summary_per_Pic), sd=sd(Summary_per_Pic))))
names(Germination_per_Treatment) <- c("Treatment", "Germination.rate",
"Stdev")
```

```
Structures_per_Treatment <- do.call(data.frame, aggregate(Summary_per_Pic[, c(9:11)], by=list(Treatment=Summary_per_Pic$Treatment), function(Summary_per_Pic) c(mean=mean(Summary_per_Pic, na.rm=TRUE), sd=sd(Summary_per_Pic, na.rm=TRUE))))
names(Structures_per_Treatment) <- c("Treatment", "Percentage_Haust", "Stdev_Haust", "Percentage_Int", "Stdev_Int", "Percentage_Rad", "Stdev_Rad")
Structures_per_Treatment <- reshape(Structures_per_Treatment, idvar=c("Treatment"), varying=2:7, sep="_", direction="long")
names(Structures_per_Treatment) <- c("Treatment", "Structure.Type", "Structure.Percentage", "Stdev.of.Percentage")
Structures_per_Treatment <- Structures_per_Treatment[order(Structures_per_Treatment$Treatment, Structures_per_Treatment$Structure.Type),]
rownames(Structures_per_Treatment) <- NULL


*Create germination and structure type graphs (of all treatments!)*
Germination_Graph <- ggplot(data=Germination_per_Treatment, aes(x=Germination_per_Treatment$Treatment, y=Germination_per_Treatment$Germination.rate))+geom_bar(position="dodge", stat="identity", fill="lightskyblue")+geom_errorbar(aes(ymin=Germination_per_Treatment$Germination.rate - Germination_per_Treatment$Stdev, ymax=Germination_per_Treatment$Germination.rate+Germination_per_Treatment$Stdev), width=0.2, position=position_dodge(0.9))+theme(panel.grid.major.x=element_blank(), axis.text.x=element_text(angle=-45, hjust=0, size=8))+xlab("Treatment")+ylab("Average germination (in %)")+scale_y_continuous(breaks=seq(0,100,10), limits=c(0,100))
print(Germination_Graph)
Structure_Graph <- ggplot(data=Structures_per_Treatment, aes(y=Structures_per_Treatment$Structure.Percentage, x=Structures_per_Treatment$Treatment, fill=Structures_per_Treatment$Structure.Type))+geom_bar(stat="identity")+scale_y_continuous(breaks=seq(0,100,10))+theme(axis.text.x=element_text(angle=-20, hjust=0, size=8))+xlab("Treatment")+ylab("Average structure frequency (in %)")+labs(fill="Structure Type")+scale_fill_brewer(palette="Blues")
print(Structure_Graph)
```

Conclusion

Strigolactone research in parasitic plants has surged since discovery of their roles as signals for AM fungi and plant hormones. Technological advances have led to high-throughput bioassays that expedite SL purification, structure determination, and analysis. Metabolomics approaches have enhanced our understanding of the involvement of stimulants and inhibitors in host/parasite interactions. Given their complex biochemistry and crucial role as germination stimulants, it is likely that

some *Striga* resistance gained through mutagenesis may involve SLs. The effectiveness of reduced *Striga* germination stimulant activity as a resistance mechanism through altered SL profiles in host root exudates must be balanced with potential fitness costs such that a net productivity gain is realized in *Striga* prone environments. The methods described in this chapter and others in this book can help determine the underlying mechanisms of *Striga* resistance gained through mutation breeding. Informed deployment in combination with good management can avoid the emergence of virulence in parasite populations, a risk of reliance on single-gene based resistance.

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Efficiency Enhancing Technologies

Identification of Closely Related Polymorphisms with *Striga* Resistance Using Next Generation Sequencing

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Abstract Molecular markers are powerful tools to enhance speed of breeding. Recent advances of sequencing technology allow us to identify most polymorphisms in a genome. Whole genome resequencing of an F2 segregating population can identify those polymorphisms that co-segregate with a mutant phenotype, which theoretically include the gene responsible for *Striga* resistance gained through mutagenesis. This protocol explains the isolation of high-quality genomic DNA for sequencing and step-by-step procedures for single nucleotide polymorphism (SNP) identification from bulked F2 sequences.

Keywords Bulked segregants · SNP detection · Next-generation sequencing · Linux

Introduction

Although breeding for resistant lines is cost-effective method for *Striga* control, the resistance or tolerance traits gained through mutagenesis often appear with unfavorable traits such as decreased yields. To avoid this problem, crossing resistant mutants with elite cultivars and screening the subsequent progeny for the plants retaining the *Striga* resistance without unfavorable traits are necessary steps. If the gene in which

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the causal mutation can be identified, an allele specific marker can be developed to track the *Striga* resistance gained through mutagenesis. Development of molecular markers co-segregating with the resistance trait will dramatically accelerate selection during introgression of the gained *Striga* resistance into elite cultivars. Genome sequencing of bulked *Striga* resistant F2 progeny of the mutant line backcrossed with the wildtype will provide candidate loci that are actually responsible for the mutant phenotype or the information of the closely linked markers (Abe et al. 2012). Whole genome sequences of many cereal species including sorghum, rice and maize are publicly available (Matsumoto et al. 2005; Paterson et al. 2009; Jiao et al. 2017), and these can be used as the mapping reference. Combined with information gained from characterizing the resistance mechanism from the screening protocols described in Chapters "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum" and "Histological Analysis of *Striga* Infected Plants", key details about the cereal/*Striga* association can be elucidated leading to possible further gene targets for improving *Striga* resistance.

One caveate about this approach is that gamma rays can cause other types of mutations besides single nucleotide changes that result in the SNP detected through the bioinformatics methods described in this chapter. From Chapter "Physical Mutagenesis in Cereal Crops", gamma rays are known to cause larger structural variations from chromosome breaks and activation of transposable elements in addition to single nucleotide variations and indels (Nielen et al. 2018; Yang et al. 2019). These larger genome structural variations may be missed in alignment of next generation sequence and the particular analysis tools described in this chapter.

Protocols

Plant materials. An advanced (M4 or later) verified *Striga* resistant mutant line should be backcrossed with the original (unmutagenized) parental wildtype line. The resulting BC1F1 should be *Striga* susceptible if the gained resistance through mutagenesis is inherited through recessive alleles. Confirm the phenotype of F1 plant by co-culture with *Striga* using the appropriate protocols described in Chapters "An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum" or "Histological Analysis of *Striga* Infected Plants". Self-pollinate the phenotyped F1 to obtain a large F2 population. Phenotype around 200 individual F2 plants by the protocol used to determine *Striga* reaction of the original mutant line and F1. Proper Mendelian segregation for a single recessive mutation is a key for successful SNP identification. Aim to collect tissue from 30–50 mutant-phenotype (*Striga* resistant) F2 plants to bulk for sequencing. As a control, also collect tissues from the wildtype parental line in a separate sample for DNA selection, and at least one (even more are better) *Striga* susceptible mutant line(s) from the original mutagenesis from which the *Striga* resistant mutant was independently isolated. These susceptible siblings serve as useful controls in addition to the parent plants and appeared to largely facilitate the identification process of SNPs responsible for *Striga* resistance trait. The end

goal is to determine the causal mutation resulting in the gained *Striga* resistance from mutagenesis, or at least a SNP that reliably co-segregates with the reistant phenotype. The protocols for actual whole genome sequencing is not covered as this will vary with the platform used. It is assumed here that a next generation sequencing platform (e.g. Illumina) on bulked DNA is used for subsequent alalysis.

High quality DNA extraction using CTAB. Good whole genome sequence starts with high quality DNA. The reagents and equipment needed for DNA extraction include:

2× CTAB buffer

2% CTAB (Hexadecyltrimethylammonium bromide, H6269, Sigma)
100 mM Tris-Cl (pH8.0)
20mM EDTA (Ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt dihydrate,

345-01865, Wako-chemical, prepare 0.5M stock with pH8.0)

1.4 M NaCl
2% PVP (polyvinylpyrrolidone mw 360,000)
0.1% beta-mercaptoethanol (Prepare on the day of experiment.)

10% CTAB

10% CTAB
0.7 M NaCl

Chloroform
Isopropanol
TE

10 mM Tris-Cl (pH8.0)
1 mM EDTA

Qiagen Genomic-tip 100/G (cat No. 10243)
Qiagen Genomic DNA Buffer Set (cat. No. 19060) (or QBT, QC and QF buffer bottles)

Add appropriate amount of beta-mercaptoethanol to 2× CTAB buffer before starting the experiment. Preheat 2× CTAB buffer at 70 °C. Prepare 60 °C shaking incubator. Prepare hot stirrer for mixing samples.

Harvest a few young leaves from each F2 individual exhibiting the mutant (*Striga* resistant) phenotype and mix them to make 1–5 g samples. Grind tissues under liquid nitrogen. Add three times (v/w) volume of 2× CTAB buffer to the sample and immediately mix them by stirring with a magnetic stirrer on a hot stirring plate. Do not allow the samples to melt. Transfer samples to a 50 mL falcon tube. Shake gently at 60 °C for 40 min. Add 1× volume (v/v) of chloroform. Gently mix the sample with chloroform in a rotator at RT (room temperature) for 10 min. Centrifuge at 3500 rpm for 20 min at RT. Transfer aqueous phase to a new tube. Add 0.1× volume (v/v) of 10% CTAB. Add an equal volume of chloroform. Gently rotate the tube for 10 min.

Centrifuge at 3500 rpm for 20 min. Transfer aqueous phase to a new tube using a glass Pastuer pipet. Add an equal volume of isopropanol. Mix very gently. Centrifuge at 10,000 rpm for 30 min. Discard the supernatant, keep the pellet. Wash pellet with 2 mL of 70% ethanol. Centrifuge at 10000 rpm for 10 min. Discard the supernatant, keep the pellet. Dissolve the pellet in 500μL TE. Measure the concentration on a spectrophotometer (e.g. Nanodrop) and fluorophotometer Qubit (ThermoFisher). Add RNaseA (Qiagen) to a final concentration of 0.1 mg/mL. Incubate at 37 °C on a heating block. Add Proteinase K (Qiagen) to a final concentration of 1 mg/mL and incubate at 56 °C for 30 min. Equilibrate the Qiagen Genomic-tip 100/G with 4 mL QBT buffer. Add 10× volume of QBT buffer (from Qiagen Genomic-tip) to the sample (5 mL to 500 μL sample) and mix thouroughly with a vortex for 20 s at a maximum speed. Centrifuge the sample at 10,000× gravity for 10 min. Load supernatant to Qiagen Genomic-tip 100/G. Wash column twice with 7.5 mL QC. Set the DNA collection tube. Elute DNA with 5 mL QF and precipitate the DNA by adding 3.5 mL isopropanol. Centrifuge at 10,000× gravity for 30 min at 4 °C. Discard the supernatant, keep the pellet, and rinse it with 70% ethanol (2 mL). Centrifuge at 10,000× gravity for 10 min, discard the supernatant and air dry the pellet. Dissolve the pellet in TE buffer to become 100–1000 μg/μL of DNA. Check the DNA quantity and quality with spectrophotometer (e.g. Nanodrop) and fluorophotometer Qubit (ThermoFisher). Abs260/280 needs to be 1.8–2.0. The concentration measured by a spectorophotometer and Qubit should have similar values. Finally, check the DNA quality by electrophoresis in 0.5% agarose gel.

SNP identification using CLC genomic workbench. The genomic DNA of bulked F2 plants with mutant phenotype is isolated and sequenced with an Illumina or equivalent sequencer with paired-end and with a read length of 150 bp. The total read number should be equivalent to the coverage 30–50. The resulting files are in fastq format. To remove unspecific SNPs, either wild type parent or unrelated mutant genomic DNA should be sequenced at the same condition as the targeted mutant F2. While it is optional, collecting genomic DNA from the susceptible siblings of each mutant is recommended. As a reference sequence, the most recent genome sequences with annotation should be downloaded from a relevant website. The sequence file is probably in fasta format, and the annotation file is in gff or gtf formats. The following is an example of using CLC Genomic Workbench software (ver. 12.0.3):

Import reference genome fasta file with function of "Import" → "Fasta High-Throughput Sequencing Import". Import GFF annotation track with function of "Import" → "Tracks". In the next page, choose the right file type and select an annotation file (gff or gtf).

Import F2 bulk genome sequence data with function of "Import" → "Illumina High-Throughput Sequencing Import". Check "paired reads" in General options and "Paired-end (forward-reverse)" in Paired-end information. Check "Remove failed reads" in Illumina options. Choose right version of Quality score (Fig. 1). The non-mutated wild type genome sequence or unrelated mutant sequence data are also imported as above.

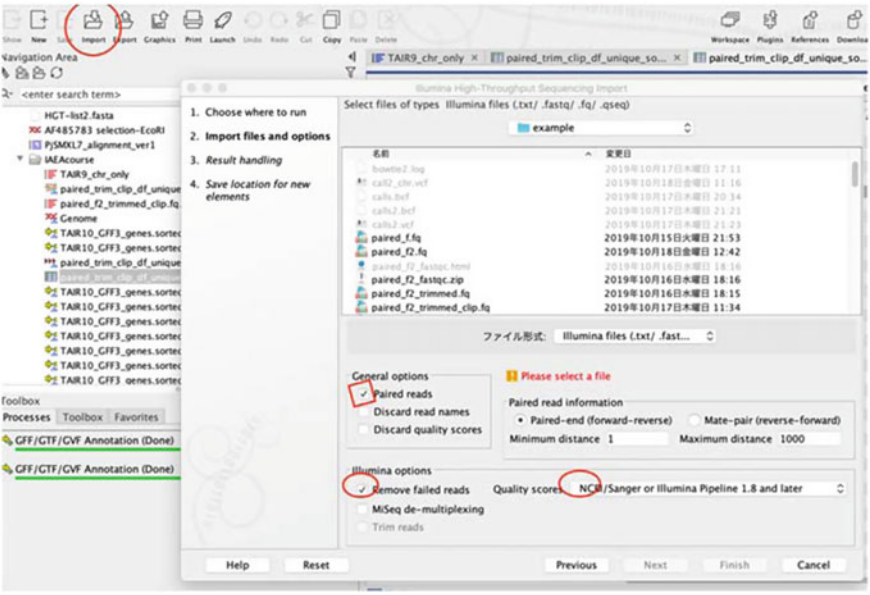

Fig. 1 Import function for the Illumina sequence reads in CLC genomic workbench. Quality score setting can be different depending on the sequencing protocol

For quality trimming of the sequence reads, check the raw data quality using "Toolbox" → "Prepare Sequencing Reads" → QC for Sequencing Reads. Trim the low-quality reads with "Toolbox" → "Trim Reads" function with quality score threshold 0.05, minimum read length 50, trimming 5 nucleotide of 5' sequence. Recheck the quality after trimming.

For mapping the quality trimmed reads, choose "Toolbox" → "Resequencing Analysis" → "Map Reads to Reference" (Fig. 2). Choose the imported Illumina data and the reference sequence for mapping. In the next page, set the value for mapping. In general cases, Match score = 1, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Length fraction = 0.6 and Similarity fraction = 0.9 can be applied (Fig. 2). Conduct the same analysis for the parental wild type (or unrelated mutant and susceptible siblings) sequence.

Variant calling. To identify unspecific SNPs, preform variant calling with low stringency filter in the control samples including the wild type parent, unrealted mutants and/or susceptible sibling. Choose "Toolbox" → "Resequencing Analysis" → "Variant Detection" → "Basic Variant Detection" and set Minimum Coverage, Minimum Counts and Minimum Frequency. We use Minimum Coverage 2, Minimum Counts 2 and Minimum Frequency 5% but these threshold should be adjusted according to your samples (Fig. 3). Low stringency SNPs called with this process will be used as background (noise) SNPs for variant filtering described below.

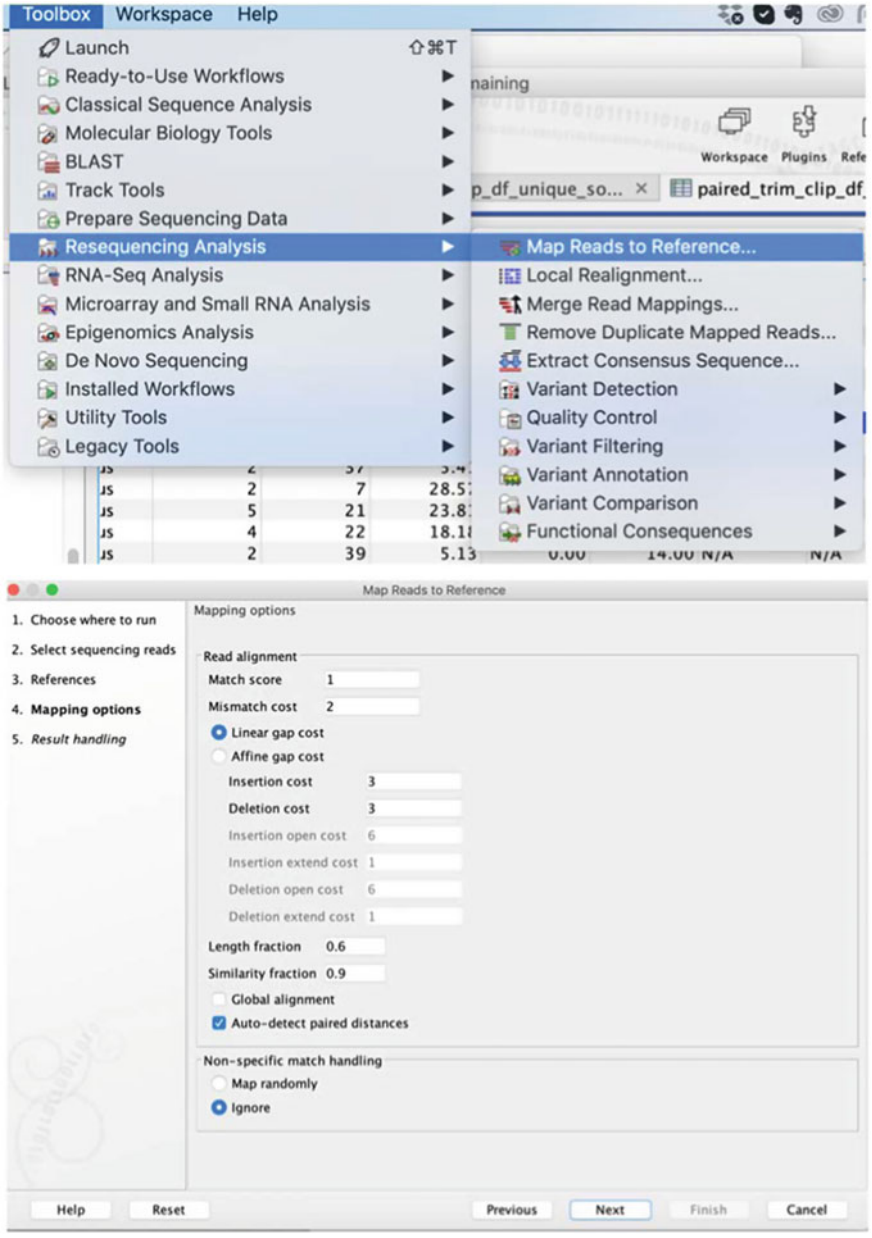

Fig. 2 Mapping of the sequence reads on the imported reference genome

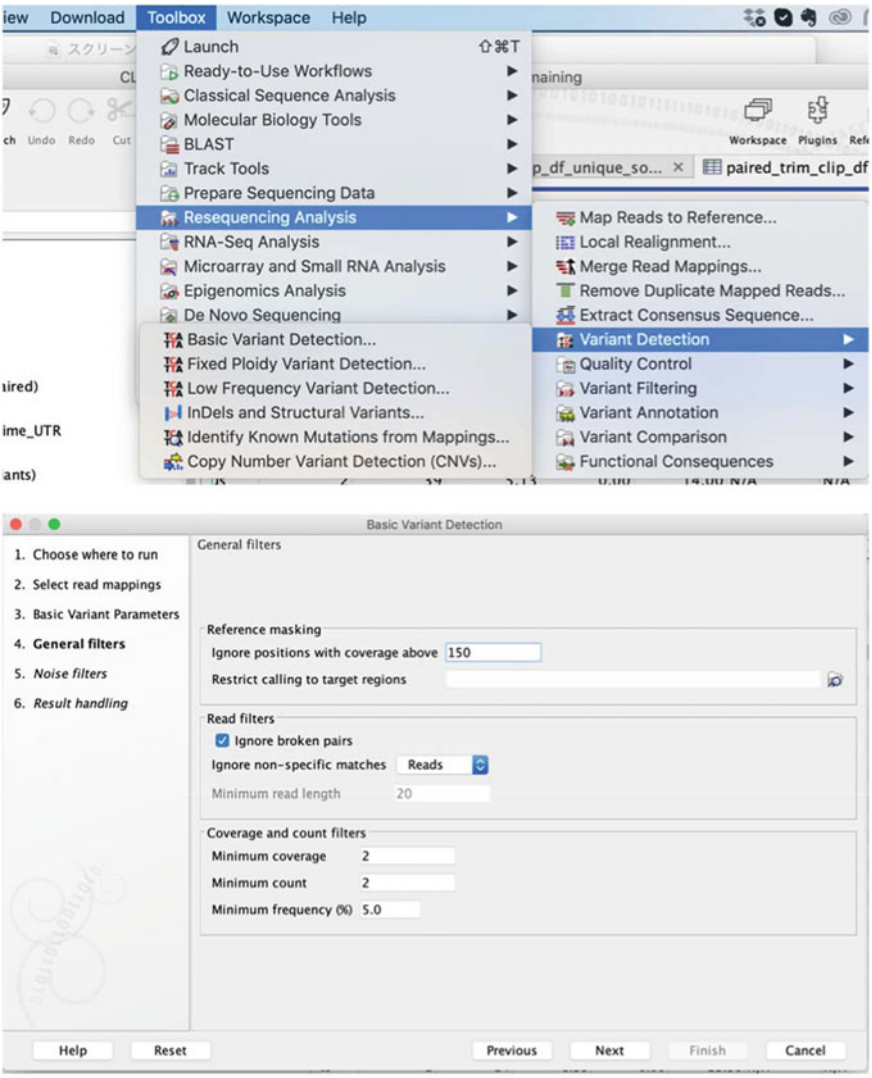

Fig. 3 Example of variant calling with a low stringency filter

For the identification of high stringency SNPs, perform variant calling with a high stringency filter. Choose "Toolbox" → "Resequencing Analysis" → "Variant Detection" → "Fixed Ploidy Variant Detection". Choose F2 bulk sequence mapping results. Set the filtering parameters. We use Minimum Coverage 6, Minimum Counts 6 and Minimum Frequency 90%, but these settings should be adjusted according to the samples (Fig. 4).

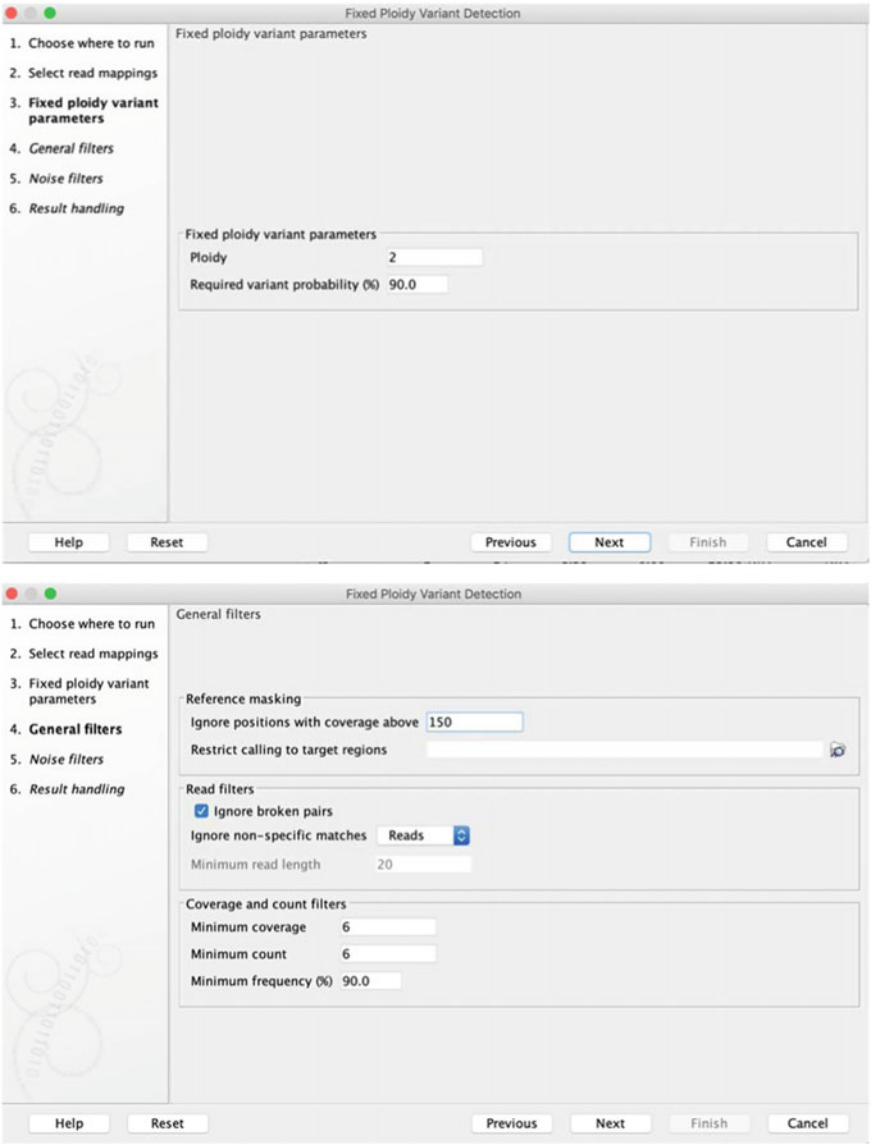

Fig. 4 Example of variant calling with a high stringency filter

Variant filtering. Perform variant filtering to identify specific SNPs for the targeted F2 mutant bulk. Choose "Toolbox" → "Resequencing Analysis" → "Variant Filtering" → "Filter against Known Variants". Choose high stringency variant detection track, and choose low stringency variant tracks as Known Variant Track. Select filter option of "Keep variant no exact match found in tracks of known variants" (Fig. 5).

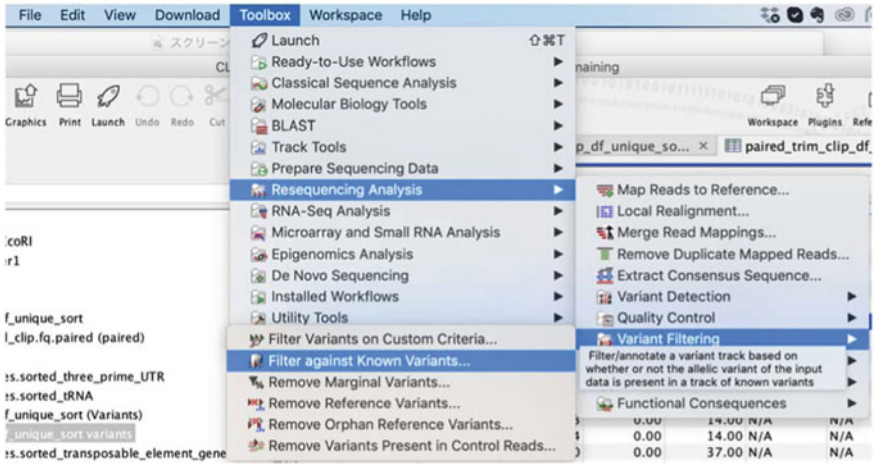

Fig. 5 Variant filtering for subtraction of non-specific variants to identify unique SNPs

Annotation of the unique SNPs. Annotate the identified unique SNPs against gene annotation of the genome. Select "Toolbox" → "Resequencing Analysis" → "Functional consequences" → "Amino Acid Changes". Select CDS tracks and mRNA tracks imported at step 1. Include upstream and downstream flanking positions of 5000 and 2000 respectively at "Flanking" option to include promoter or 3' UTR mutations. Then, the annotation table will be obtained. Check manually for the sequence alignments of the detected non-synonymous SNP regions. Sometimes, failure of alignments results in the false position detection of the unique SNPs. Carefully remove such non-specific SNPs.

Example of application and validation of the utility of the protocol. An example of mutated gene identification using the method described above is available in Cui et al. (2020) using the parasitic plant *Phtheirospermum japonicum.* Sequencing of approximately 40 times coverage from F2 plants as well as parental mutant lines identified approximately 450 thousand SNPs respectively. Filtering out the non-specific SNPs that were found in low stringency filter from unrelated mutant lines reduced the SNP number to less than 2000 in F2 progeny. Selection of the non-synonymous mutation from the unique SNPs identified two candidate genes for the causal mutation.

SNP detection using open-source scripts. As an alternative approach, SNP detection can be performed with open-source scripts. This section explains how to perform SNP detection using open-source software mainly using a Linux computer.

Quality of sequence reads can be visualized by fastqc. fastqc is available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ under GPL v3 license. This script works at Linux, Windows and Mac architectures. Executables can be directly downloadable from the above site. Fastqc is a java application and suitable Java Runtime Environment (JRE) installation is prerequired (Fig. 6).

Trimmomatic (Bolger et al. 2014) is useful for removing adaptor sequences and quality trimming with flexible setting. This is also made in java platform and works with Linux, Windows and Mac. The binary files and manual can be downloaded from http://www.usadellab.org/cms/?page=trimmomatic. An example command for trimmomatic quality filtering follows while the adaptor sequence file and valuable setting should be modified according to the quality requirement.

```
>java -jar trimmomatic-0.39.jar PE -threads 4 -phred33 -trimlog
trim.log -basein YOUR_INPUT_1.fq.gz -baseout OUTPUT_name
ILLUMINACLIP:TruSeq2-PE.fa:2:30:10:2:keepBothReads HEADCROP:15
LEADING:30 TRAILING:30 SLIDINGWINDOW:5:20 MINLEN:50 > test.log
2>&1 &
```

This command outputs 4 files with the prefix baseout followed by _1P, _2P for paired sequences and _1U and _2U for unpaired sequences. Standard out contains percentage of surviving sequences.

Read mapping using bowtie. There are numbers of mapping software available with different performance. Here we explain mapping process using bowtie2 (Langmead and Salzberg 2012).

The Bowtie2 script is available at http://bowtie-bio.sourceforge.net/bowtie2/index.shtml.

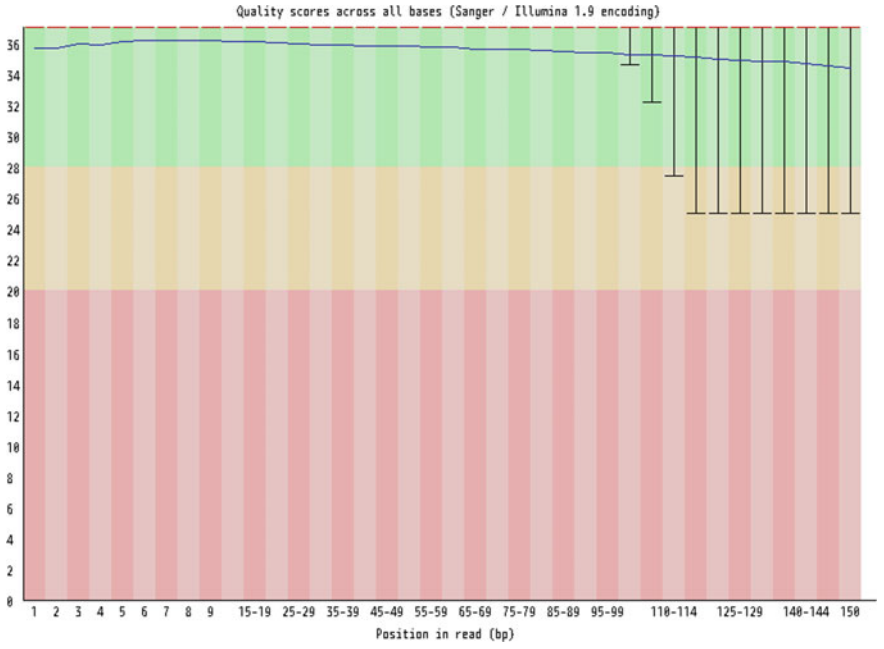

Fig. 6 Examples of fastqc output. Quality scores across all bases (top) and sequence contents across all bases (bottom)

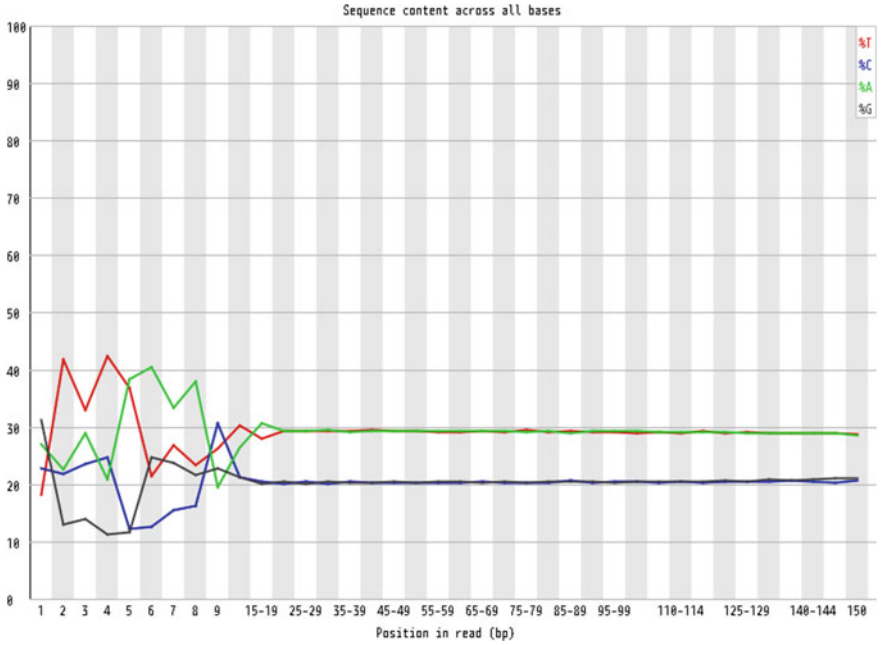

Fig. 6 (continued)

Download and install according to the instructions. Make an index from your reference genome (fasta format) with the following command.

```
>bowtie2-build reference_genome.fasta INDEX_name
```

Run mapping with bowtie2.

```
>bowtie2 -x INDEX_name -1 forward.fastq -2 reverse.fastq -p number_
of_cpu -S OUTPUT.sam >bowtie.log 2>&1 &
```

Extract uniquely mapped reads from sam file.

```
>grep -v "XS:" OUTPUT.sam> OUTPUT_unique.sam
```

Sort and convert the sam file to binary bam file using samtools (available from http://samtools.sourceforge.net).

```
>samtools sort -@ thread_number -O bam -o OUTPUT_unique.bam OUTPUT_
unique.sam
```

Indexing the bam file.

```
>samtools index OUTPUT_unique.bam
```

Calculate coverage of the mapping.

```
>samtools depth -aa OUTPUT_unique.bam >OUTPUT_unique.depth
```

You will get the table file with depth of each nucleotide. Calculate average coverage from the depth file using awk command.

```
>cat OUTPUT_unique.depth | awk '$3>0{print} | awk '{sum+=$3} END
{print sum/NR}' &
```

Alternatively, you can also calculate the average coverage using excel or R Plot the coverage using R to investigate whether there is any large deletion in the sequence. Because the data set is too large, split the depth file to each chromosome.

```
>awk '$1 == 1 {print $0}' OUTPUT_unique.depth > Chr1.depth
```

Start R program. Use library reshape and ggplot2.

```
>R
```

In R console,

```
>library(reshape)
>library(ggplot2)
>OUTPUT.Chr1<-read.table("Chr1.depth",header=FALSE,sep
="¥t",na.strings="NA")
```

Rename header.

```
>OUTPUT.Chr1<-rename(OUTPUT.Chr1,c(V1="Chr",        V2="locus",
V3="depth"))
```

If you want to visualize each genotype together in a single plot, add genotype column.

```
>OUTPUT.Chr1<-transform(OUTPUT.Chr1, genotype="WT")
```

Change genotype to suitable setting (WT, M4 or F2 etc.). Repeat the same for different genotypes. Because entire chromosome is too large to visualize in one plot, extract a subset of sequence. Extraction can be one nucleotide in 50 bp, or a certain region from the chromosome.

```
>Chr1_sub < -OUTPUT.Chr1[seq(1,nrow(OUTPUT.Chr1),by =
50),] #extract every 50 bp.
>nrow(Chr1_sub) #count number of row.
```

Repeat the same procedure for different genotypes. Row bind each genotype result.

```
>Chr1_bind_sub<-rbind(Chr1_sub_WT,Chr1_sub_F2)
```

Plotting.

```
> g<-ggplot(Chr1_bind_sub,aes(x=locus,y=depth,colour=genotype))
> g<-g+geom_point()
> g<-g+facet_grid(genotype ~ .)
```

Set the maximum coverage to plot because some nucleotides have a huge coverage value that interfere with proper visualization of the plot. The following limits the maximum coverage up to $\times 50$.

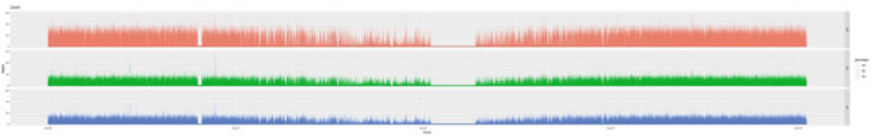

Fig. 7 Example of coverage plotting of Chr9 in *Sorghum bicolor*

```
> g<-g+ylim(c(0,50))
```

Add title of the chart.

```
>g<-g+ggtitle("Chr01")
```

Adjust width and height of the chart by changing width and height option below (Fig. 7).

```
> ggsave("Chr1.png",g,width=20, height=6,dpi=150)
```

Extract SNPs from the mapping data using bcftools (available from http://www.htslib.org/doc/bcftools.html). Call SNPs by bcftools.

```
> bcftools mpileup -Ou -f reference.fasta --max-depth 250 -a FORMAT/
AD OUTPUT_unique_wt.bam | bcftools call -mv -Ob |bcftools view >
OUTPUT_unique_wt.vcf
```

Do the same for other genotypes. Filter the SNPs using quality score and allele frequency. For low stringency filter apply allele frequency threshold 0.05.

```
> bcftools filter -i 'QUAL > 20 && DP > 2 && (DP4[2]
+ DP4[3])/(DP4[0] + DP4[1] + DP4[2] + DP4[3]) > 0.05'
OUTPUT_unique_wt.vcf > OUTPUT_unique_wt_0.05.vcf.
```

High stringency filter for allele frequency threshold 0.8.

```
> bcftools filter -i 'QUAL>20 && DP>10 && (DP4[2]+DP4[3])/
(DP4[0]+DP4[1]+DP4[2]+DP4[3])>0.8' OUTPUT_unique_mt.vcf >
OUTPUT_unique_mt_0.8.vcf
```

Compress the vcf file.

```
> bgzip OUTPUT_unique_mt_0.8.vcf
```

Index the vcf file.

```
> bgzip index OUTPUT_unique_mt_0.8.vcf.gz
```

Intersect the SNPs from low stringency filter from those with high stringency filter.

```
>bcftools isec bgzip index OUTPUT_unique_mt_0.8.vcf.gz OUTPUT_
unique_wt_0.05.vcf.gz -p output -C
```

Output file 0000.vdf is generated in the folder specific by –p. Check the passed SNPs number.

```
> grep "PASS" 0000.vcf |wc -l
```

Depending on the extracted SNP number, adjust the filter setting for quality and allele frequency. Annotate unique SNPs using SnpEff. SnpEff is available from http://snpeff.sourceforge.net. Install SnpEff according to the instruction. Download database from SnpEff database. Search the reference genome in the database. This is an example of sorghum.

```
> java -jar snpEff.jar databases| grep -i "sorghum"
```

Download the reference from the indicated database.

```
>wget    http://downloads.sourceforge.net/project/snpeff/databa
ses/v4_3/snpEff_v4_3_ENSEMBL_BFMPP_32_375.zip
```

If the reference genome is not available in the SnpEff database, make a reference database. Please note that annotation for the same species could sometimes be different between EMBL and Phytozome. This is an example to make sorghum_v3.1.1 database from the Phytozome sorghum genome.

```
>mkdir snpEff/data/sorghum_v3.1.1
>mv sorghum_referemce.fasta snpEff/data/sorghum_v3.1.1/
>mv sorghum_referemce.gff snpEff/data/sorghum_v3.1.1/
```

Edit snpEff.config to adjust reference and gff. Build the new database.

```
> java -jar snpEff.jar build -gff3 -v sorghum_bicolor_
v_3_1_1.
```

Check the database.

```
> java -jar snpEff.jar databases |grep -i "sorghum".
```

Annotate the SNP location.

```
> java -Xmx4g -jar snpEff.jar -c snpEff/snpEff.config Sorghum_
bicolor_v_3_1_1 0000.vcf >annotated.vcf
```

Effects of mutation on the protein sequence are labelled according to their predicted severity (e.g., "MODERATE" or "HIGH"). The mapping bam file and vcf files can be visualized by IGV (available from http://software.broadinstitute.org/software/igv/) (Fig. 8).

Conclusion

Next generation sequencing is a powerful tool to identify mutant causal genes or closely related markers. Commercialized software or free open-source script are available to perform the SNP detection. The identified SNPs can be converted into PCR markers such as CAPs or dCAPs to analyze mutant populations. Functional validation should be followed after SNP identification. In the case of larger genome

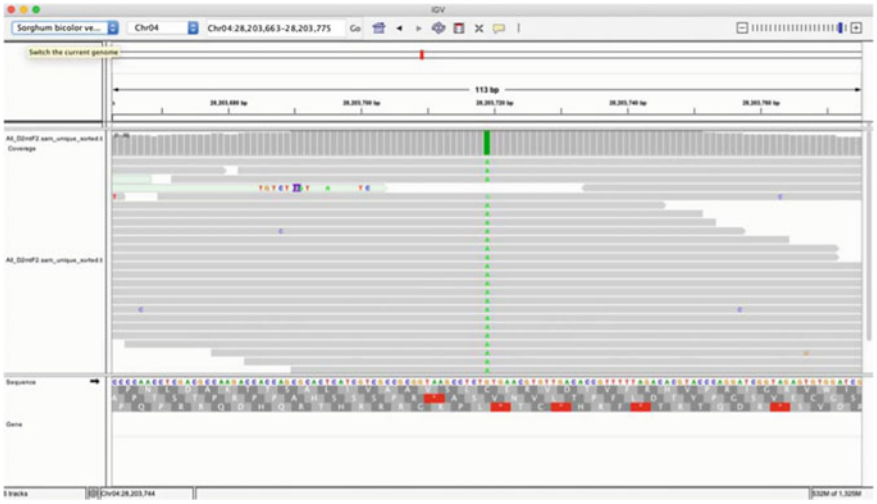

Fig. 8 SNP visualization by IGV

structural changes caused by physical mutagens, alternative sequencing and bioinformatics methods may be needed to identify causal mutations underlying gained *Striga* resistance.

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Rapid Cycling and Generation Advancement for Accelerated Mutation Breeding in Sorghum

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Abstract Rapid cycling techniques have become preferred methods in recent years to speed up the plant breeding process, especially for plant varieties recalcitrant to *in vitro* tissue culture. Manipulation of plant growth management of components such as pot size, irrigation, nutrition and light/day length can substantially accelerate the plant growth cycle from seed to harvest. In this chapter, we describe how to shorten the generation time of sorghum to have 4–6 generations per year to speed the plant breeding process including mutation breeding for resistance to *Striga*.

Keywords Rapid cycling · Accelerated breeding · Sorghum · Embryo rescue

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench), is among the global top five grain crops, widely consumed as food in many parts of the world, especially Africa. In contrast to maize and rice, sorghum can remain productive under low input agriculture in areas of limited irrigation and soil fertility. Recent abrupt climate changes and spread of the parasitic weed *Striga* spp. have constrained crop production in sub-Saharan Africa and semi-arid tropical regions of Asia particularly. This situation has compelled plant breeders to produce climate change-resilient crops, which can withstand broad-spectrum biotic and abiotic stresses. New sources of genetic variation are vital for

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crop improvement and achieving sustainable yield increases. However, limited variation for traits that mitigate the impact of these productivity challenges within some sorghum populations make the breeding gains difficult (Atera et al. 2012; Weltzien et al. 2018). Mutagenesis can expand the genetic base for crop resilience to environmental stresses. This was the basis for the Coordinated Research Project (D25005) launched by the IAEA in 2016 to optimize protocols for mutation breeding and efficiency enhancing technologies for resistance to *Striga* spp. in cereals for food security.

Aside from genetic resource erosion or scarcity in certain crop populations, long growing seasons can also limit rapid development and delivery of improved varieties. A number of the intervention technologies in crop management can shorten the delivery time of improved varieties. One such technology is doubled haploids (DH) which increases the efficiency of selection through accelerating fixation of desirable alleles to homozygosity thereby avoiding multiple generations of inbreeding. Doubled haploid techniques have been successfully deployed in maize (Chaikam et al. 2019) and rice (see following chapter) and therefore could streamline the process of deploying traits like *Striga* resistance gained through mutagenesis in these crops. However, for crops recalcitrant to tissue culture like sorghum, employing DH technology is severely restricted.

As an alternative to DH mitigated fixation of mutant alleles that improve *Striga* resistance in sorghum varieties, rapid cycling of selfing generations can be employed. Inbreeding in sorghum is fairly easy since it is a self-pollinated crop. However, many varieties, particularly those native to *Striga* endemic agroecologies, are day-length sensitive, requiring a short photoperiod (12 h or less) to flower and/or very long growing seasons (> 180d). Recent developments enable acceleration of sorghum growth cycles through the use of glasshouses and growth chambers with controled temperature, photoperiod, pot size, and irrigation combined with *in vitro* embryo rescue techniques. Perhaps owing to sorghum's ability to produce grain in stressful environments, horticultural management that limits rooting space and other resources generally speeds generation time (seeds sown to seeds harvested). Rapid cycling techniques can be adapted to varieties with both neutral and short-day photoperiods. They can greatly reduce the conventional mutation breeding time to improved variety delivery of 10–12 years (Forster et al. 2014; Ghanim et al. 2014; Yan et al. 2017; dan Anisiyah 2020). This chapter describes such rapid cycling protocols for sorghum combining growth management with embryo rescue for accelerating mutation breeding for *Striga* resistance.

Protocols

Plant materials. Nine different sorghum varieties with varying growth season lengths were originally collected from Sudan Agricultural Research Corporation germplasm. These are named Abu 80, Arfagadamak, Arossarimal, Bashair, Botana, Tabat,

Mugud, Wad Ahmed and Yruasha. Table 1 shows days to flowering, days to maturity and a typical number of seeds/panicle for each of these varieties under normal agricultural condition in Sudan (Eltahir et al. personal communication).

Plant growth conditions. Germinate sorghum seeds of selected genotypes/varieties in petri dishes and transplant germinated seeds after 5–7 days. Recently harvested seeds can be sown directly in soil in containers such as plastic boxes, trays with small cells (5 × 5 × 5 cm), small pots (9 × 9 × 5 cm) containing the soil mixture: one part sand to one part peat moss (Fig. 1).

Initially, set growth chamber or glasshouse temperature to 25 °C with a 24 h photoperiod (use supplemental light in glasshouse), at 60% relative humidity (RH) until seedlings reach the 4–6 leaf stage. Note that these conditions depend on the sorghum genotype and the control capacity of the growing facility. Night time temperatures should not fall below 22 °C. Keep the seedlings regularly irrigated with tap water in 2–3 day intervals or when needed and fertilize weekly with full strength Hoagland's solution (Hoagland and Arnon 1950). Ensure that the plants remain free from pests and diseases. After about four weeks from planting, change the day length to shorter than 12 h to stimulate flowering in short-day varieties. For photoperiod sensitive varieties, growth chambered plants will need 12 h days from approximately four weeks until they are six weeks old to induce flowering. If photoperiod sensitive plants are grown in a glasshouse, shading during this developmental period that completely excludes light (e.g., putting a box over the tray or black cloth over a bench) are required to achieve 12 h day length in the months between the Spring and Winter equinox to induce flowering. Cover flowering heads with an appropriate sized paper bag to ensure self-pollination. Record flowering dates and physiological maturity to estimate the generation time. Collect pre-mature heads (10, 15, 20 days after anthesis) and proceed to in vitro laboratory for embryo rescue for testing the best time of collection for immature embryos as an option to further shorten the generation times. Keep the plants until physiological maturity and harvest the seeds for planting

Table 1 Days to flowering, days to maturity, number seeds per head and sensitivity to photoperiod of the tested varieties under the normal growing condition at different region in Sudan

Variety	Days to 50% flowering	Days to maturity	Number of seeds/head (×1000)	Sensitivity to photoperiod
Abu 80	60–65	105–119	1.3–1.5	Insensitive
Arfagadamak	58–62	109–124	1.6–2.4	Sensitive
Arossarimal	57–63	116–129	1.4–1.6	Sensitive
Bashair	58–65	115–140	1.3–1.8	Sensitive
Botana	55–64	112–137	1.5–2.0	Sensitive
Tabat	64–67	123–142	1.7–3.2	Sensitive
Mugud	70–74	149–173	0.8–1.3	Sensitive
Wad Ahmed	66–69	133–147	1.6–3.3	Sensitive
Yruasha	61–65	119–138	1.3–1.5	Sensitive

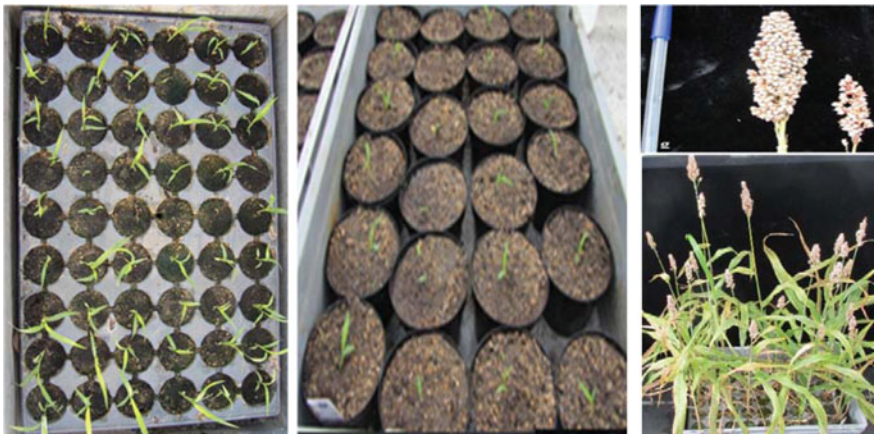

Fig. 1 Representative photos for seedlings of sorghum in small trays (**a**) and round small pots (**b**) for rapid generation cycling (**c**) shows plants at harvesting stage and representative bigger (left) and smaller (right) heads harvested from round small pots and trays, respectively

directly in the next cycle if there is no in vitro culture facility for embryo rescue (Fig. 1). Repeat the above cycle consecutively until sufficient inbreeding (mutant alleles fixed) is achieved.

Premature harvesting of heads and embryo rescue. Harvest immature heads after 10, 15 and 20 days from anthesis and remove immature sorghum seeds (Fig. 2). Inside a laminar flow hood, surface sterilize the harvested immature seeds with 70% ethanol for two minutes and 40% Chlorox (2.1% NaOCl) for five minutes. Rinse the seeds after each of the disinfection steps three times with sterile distillated water. Remove immature embryos from the caryopses with a sterile lancet and forceps under aseptic conditions (Fig. 2). Place the scutellum up on the surface of the growth medium in a petri dish with the embryo-axis in contact with the culture medium (see details below).

Embryo rescue and culture conditions. Culture the collected immature embryos in petri dishes containing regeneration medium with half strength MS salts (Murashige and Skoog 1962), 10 gL^{-1} sucrose, 0.8% (w/v) agar and 1% (w/v) charcoal (see preparation instructions below). Firmly seal the culture plates with Parafilm M®. Incubate these cultures in a growth chamber/incubator under 16 h light/8 h dark photoperiod, light of 500 $\mu mol\ m^{-2}\ s^{-1}$ photon flux density and temperature of 25 °C for 4–5 days or until proper germination. Transfer germinated sorghum seedlings in small trays/pot containing 1:1 peat:soil mixture (Fig. 3). Incubate transplanted seedlings in greenhouse conditions similar to those described for rapid cycling of mature seeds. Repeat to new cycle when the plants reach the optimum time for immature seed collection for embryo rescue or the physiological maturity for seed collection.

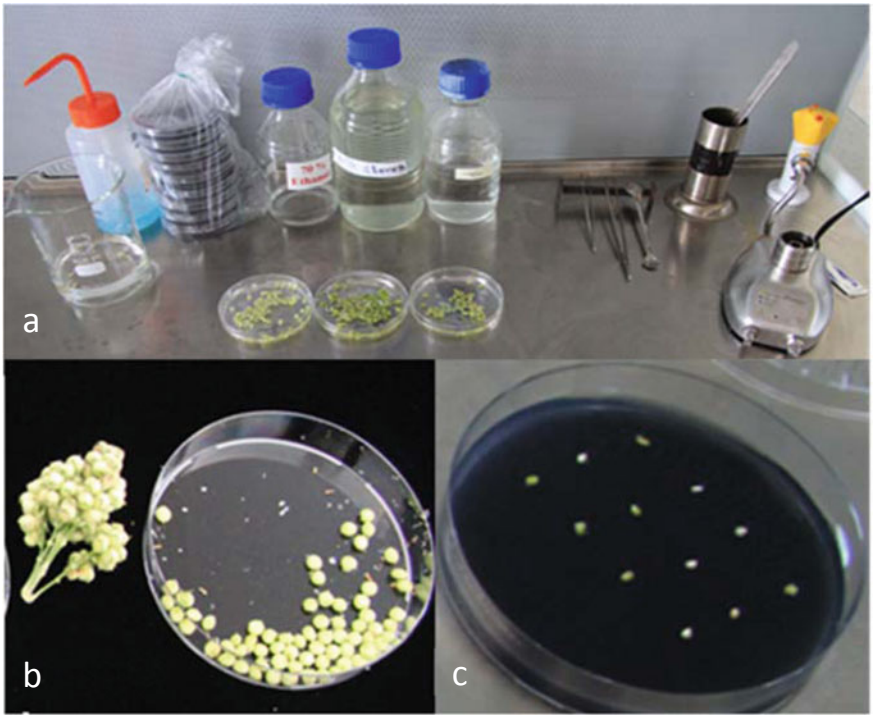

Fig. 2 Representative photos showing *in vitro* set up and sterilization solutions for embryo rescue (**a**), collected immature head and removed seeds (**b**), and aseptically removed embryos placed on culture medium for germination (**c**)

Media preparation for seedling regeneration and sterilization. Dissolve 2.15 g/L MS (Murashige and Skoog 1962) Basal Salt Mixture (Sigma M5524) and 10 g/L sucrose in 1L distillated water at room temperature. Adjust the pH to 5.7–6.0 using 1N NaOH/KOH or 1N HCl. Add 10 g/L charcoal and 8 g agar into media to solidify. Autoclave for 20 min at 121 °C at 15 psi. Distribute the autoclaved media after cooled to 50–60 °C into 90 mm diameter disposable sterile petri-dishes. Store the media at room temperature or 4 °C for prolonged storage.

Validation of the protocol. Among the nine tested sorghum varieties, the flowering time ranged between 36–47 days when grown in trays, and 42–57 days in round small pots (Table 2). Days to seed physiological maturity ranged between 65–75 days in the trays, and between 75–92 days in the small pots. The number of seeds harvested per head ranged between 25–76 seeds in trays and between 85–295 seeds in the small pots. These results indicate that in two months one could harvest sufficient seeds to proceed to the next cycle using the trays (5 × 5 × 5 cm) or small round pots (9 × 9 × 5 cm). This would allow up to 4–5 crop cycles per year. Seed germination tests at different dates from flowering indicated that within a month from harvesting, germination is more than 50% compared to 100% after 45 days from flowering (Table 3).

Fig. 3 Representative photos showing the transfer of germinated seedling from immature embryos (petri-dish inset of A) into trays with cells filled with peat:soil mixture (**a**), covering of the transplanted seedlings with a nylon cover for about 7–10 days for acclimatization (**b**), and removal of the nylon cover from acclimated seedlings for continued growth in the open environment of the glasshouse (**c**)

This suggests that further shortening is possible in the time needed for the crop cycle with early harvesting and immediate planting as only a few (theoretically just one) plants are needed to proceed from each parental plant to the next cycle. Furthermore, with intervention by embryo rescue from immature seeds at 15 days from flowering, 70–100% of the immature seeds germinate. This would further shorten the crop cycle by two weeks (Table 4).

Table 2 Effect of different pot type and management on days to flowering, days to maturity and number of seeds per head in the different sorghum varieties

Variety	Tray (5 × 5 × 5 cm)[a]			Small round pots (9 × 9 × 5 cm)[a]		
	Days to 50% flowering	Days to maturity	Number of seeds/head	Days to 50% flowering	Days to maturity	Number of seeds/head
Abu 80	40	68	53	42	75	101
Tabat	41	71	74	45	80	250
Botana	39	67	57	51	83	104
Arfagadamak	38	65	76	53	86	295
Wad Ahmed	44	75	65	55	90	315
Mugud	47	69	75	57	92	181
Bashair	42	67	25	49	85	85
Yruasha	42	70	40	57	87	317
Arossarimal	36	65	43	40	75	236

[a]Data are average of 6 plants (pots) per variety

Table 3 Germination percent of freshly harvested seeds after 30, 35 and 45 days from flowering among the nine tested sorghum varieties across trays and small round pots

Seed germination % at three intervals from flowering			
Variety	30 days (%)	35 days (%)	45 days (%)
Abu 80	53	71	100
Tabat	62	98	100
Botana	61	63	100
Arfagadamak	53	61	73
Wad Ahmed	72	97	100
Mugud	51	73	91
Bashair	58	71	100
Yruasha	42	69	100
Arossalrimal	43	62	83

Data are averages of three heads per variety

Table 4 Frequency of regeneration of plantlets from immature embryos rescued at 10, 15 and 20 days post-anthesis from nine sorghum varieties

	% Regenerated plantlets at three intervals from anthesis		
Variety	10 days (%)	15 days (%)	20 days (%)
Abu 80	58	82	63
Tabat	61	72	74
Botana	89	96	73
Arfagadamak	92	91	81
Wad Ahmed	63	83	53
Mugud	47	68	49
Bashair	49	72	52
Yruasha	62	68	48
Arossalrimal	39	97	53

Data was collected from three immature heads per variety

Conclusion

Induced mutations in sorghum by physical mutagens such as gamma and X-rays coupled with rapid cycling of generations offer promising opportunity to accelerate delivery of mutant varieties with induced resistance to the parasitic weed *Striga.* In this study, the protocol is optimized for horticultural factors such as pot size, temperature, irrigation, photoperiod and use of immature embryo rescue to accelerate propagation and time needed to complete the growth cycle (Fig. 4). The optimized protocol was validated on nine different sorghum varieties. By using small rooting areas and intervention with embryo rescue, the time needed to grow and harvest seeds/embryos was reduced from 5 to 6 months under natural conditions in the field to 2–3 months under the optimized protocol (Fig. 4). This means with the optimized protocol we can advance four to six generations per year and thus fix the mutant trait in two years and quickly advance to multi-location evaluation and varietal registration. The entire process takes up to 10–12 years under a conventional mutation breeding approach.

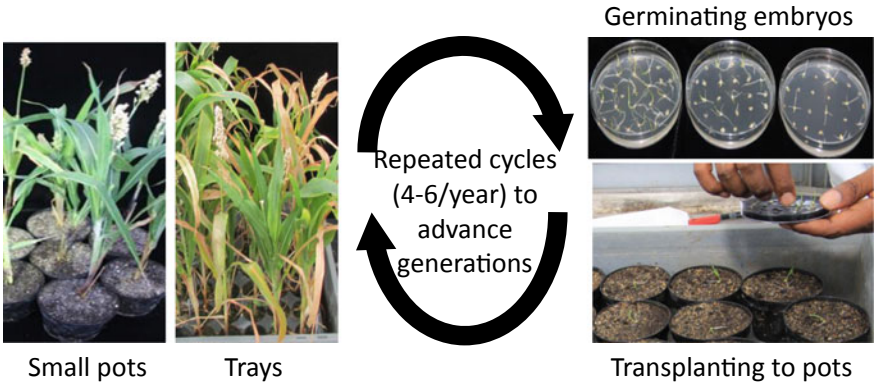

Fig. 4 Summarizes the optimized scheme for rapid generation cycling of sorghum

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Anther Culture of Rice for Haploidy Induction and Accelerated Development of *Striga* Resistant Germplasm

Ayşe Şen and Necmi Beşer

Abstract Doubled haploidy is the fastest way of achieving completely homozygous plants in one generation. It shortens the time required for the development of mutant varieties allowing rapid response against threats to sustainable food production caused by changing climate and stresses due to emerging pests like *Striga*. In this chapter, we describe step-by-step protocols to obtain doubled haploid rice with fixed novel mutant variants by anther culture from gamma ray irradiated M1 populations to accelerate mutation breeding for resistance to *Striga* in rice.

Keywords Doubled haploid · Rice · Anther culture

Introduction

The development of resistance in host species remains one of the most efficient and cost-effective ways to control infestations of parasitic plants (Press et al. 1995). With limited natural alleles conferring *Striga* resistance among cereal crops, particularly rice and maize (see Chapter "Striga as a Constraint to Cereal Production in Sub-Saharan Africa and the Role of Host Plant Resistance"), physical mutagenesis is an attractive means by which to generate new sources of host plant resistance (Ulukapi and Nasircilar 2018). As we have seen in previous chapters (Chapters "Screening for Resistance to Striga Hermonthica in Mutagenized Sorghum and Upland Rice in Burkina FASO"–"Phenotyping for Resistance to Striga Asiatica in Rice and Maize Mutant Populations in Madagascar" and "Rapid Cycling and Generation Advancement for Accelerated Mutation Breeding in Sorghum"), achieving true-breeding *Striga* resistant lines from mutagenized cereals takes several generations. The single

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seed descent (SSD) method is one of the conventional ways used for generating homozygous lines in cereal breeding. However, six generations of inbreeding are required to obtain about 98% homozygosity in the SSD method (Khound et al. 2013). Screening for *Striga* resistance cannot begin until the M2 and verification of gained resistance is usually not accomplished until the M4 or M5. Advancement to new *Striga* resistant varieties suitable for public release also involves several generations of multi-location field trials. Anther/microspore cultures are of tissue origin, based on the method to produce completely homozygous (doubled haploid; DH) plants from immature pollen grains. Doubled haploidy is the fastest way of achieving completely homozygous plants (in one generation). It shortens the time required for development of novel plant genotypes than the conventional methods that require at least 6–7 generations of inbreeding and selection. This process is the reason for the time taken, often 11–13, years between making crosses to releasing cultivars (Germana 2011). Progress in haploid/doubled haploid production, such as the rapid generation of large microspore-derived haploid populations via androgenesis, may be exploited in mutation breeding. For example, genome-fixed novel mutant variants obtained by anther culture from the irradiated M1 population can be rapidly incorporated into breeding/selection programs such as developing novel variants resistant to *Striga*. The aim of this study was to develop and apply haploid/doubled protocols via androgenesis in rice for production of mutant haploid/doubled haploid populations.

Protocols

Rice (*Oryza sativa* L. cvs. Taipei 309, Osmancık-97, TG1) seeds were irradiated at 100, 200 and 300 Gy doses by exposure to a ^{137}Cs gamma ray source in the Nordion Gamma Cell 3000 at the Elan irradiation facility of the Istanbul School of Medicine Blood Bank Center, Istanbul University, Istanbul, Turkey.

Panicle collection and pre-culture treatment. Sow the irradiated rice seeds in the field or in greenhouse pots under optimal growing conditions for rice. Collect panicles from primary tillers when the auricle distance of the flag leaf to the next leaf is 7–10 cm (Fig. 1). Remove outer leaf sheaths and ensure that the panicles are wrapped with the inner leaf sheath. Wash the boots or panicles in tap water and stand them in a plastic water bottle. Cold treat panicles at 4 °C for 8–10 days in a refrigerator or a cold room.

Determination of microspore viability and pollen stage. Crush anthers in buffer and filter them through 40 μm steel mesh. Centrifuge the homogenate at 2000 rpm for 2 min. Remove supernatant and use the pellet for the squash preparation. Add 0.1 μL of FDA (fluorescein diacetate) stock solution (5 mg of FDA in 1 mL of acetone) to 1 mL of the microspore suspension. Remove a drop to a glass microscope slide and add a coverslip. Examine slides under a fluorescent microscope using UV-light and the appropriate filter set. Viable microspores glow bright green (Fig. 2a).

Fig. 1 Photos show the optimum stage for rice panicle collection indicated by white arrow (**a**), panicles with the outer leaf sheath removed (**b**), and cold treating panicles in a refrigerator (**c**)

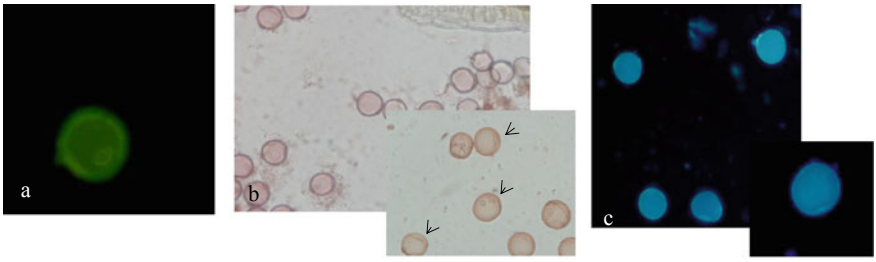

Fig. 2 2% Aceto-carmine (**a**), FDA (**b**) and DAPI (**c**) staining: black arrows indicated that uninucleated microspore cells

Determination of microspore developmental stages. Crush anthers in 2% aceto-carmine stain. Cover them with a glass slip. Observe microspore stage under a light microscope (Fig. 2b).

DAPI staining. Crush anthers in buffer and filter them through 40 μm steel mesh. Centrifuge homogenate at 2000 rpm for 2 min. Remove supernatant and use the pellet for the squash preparation. Fix 100μL of cell debris in ethanol:acetic acid (3:1) for 15 min. Centrifuge homogenate at 2000 rpm for 2 min. Remove supernatant, wash the pellet once (or twice) in 50% ethanol, and then centrifuge homogenate again between each of the washing steps. Remove supernatant and add 10 μL DAPI (4,4-diamidino-2-phenylindole) working solution [stock solution (5 mg of DAPI in 1 mL of 50% ethanol) diluted in 1 ml of 50% ethanol] and a drop of glycerol. Examine slides under the fluorescent microscope using UV-light and the appropriate filter set (Fig. 2c). Finally, according to 2% aceto-carmine and DAPI staining, select anthers having microspores at mid-uninucleate to early binucleate stage of development, as these are the most responsive to anther culture.

Surface sterilization of panicles. Surface-sterilize cold treated boots by immersion in 70% (v/v) ethanol for 2 min followed by three times wash with sterilized distilled water. Put in 40% Clorox (2.1% NaOCl) for 25 min and then wash by dipping three to four times in a series of vials filled with sterilized distilled water until the bleach smell dissipates.

Anther culture and callus induction. Place panicles on sterilized filter papers. Remove anthers aseptically with a pair of forceps and gently place in a 35 × 15 mm disposable petri dish containing 3 mL of androgenic calli induction medium [N6 (Chu et al. 1975) contains 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), gum arabic, and 40 g/L maltose, pH at 5.7–6.0 (modified by Afza et al. 2000)]. Up to 50 anthers are cultured in each disposable petri dish with a few ovaries. Firmly seal Petri dish with Parafilm M® barrier film and incubate them at 27 ± 1 °C with 12/12 h light/dark period. Observe for callus formation in three to four weeks after anther planting (Fig. 3a–d).

Media preparation for androgenic calli induction and sterilization. Dissolve 4 g/L N6 (Chu et al. 1975) basal salt mixture (Sigma C1416), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 40 g/L maltose in 1L distilled water at room temperature. Adjust the pH to 5.7–6.0 using 1N NaOH/KOH or 1N HCl. The media should be autoclaved for 20 min at 121 °C under 1.25Atm. Filter-sterilize gum arabic and then add it to the autoclaved medium cooled to 40–50 °C. Store medium at 8–10 °C.

Plantlet induction from androgenic calli. Pick up androgenic calli with a sterile pair of forceps. Transfer them into semisolid regeneration medium [MS (Murashige and

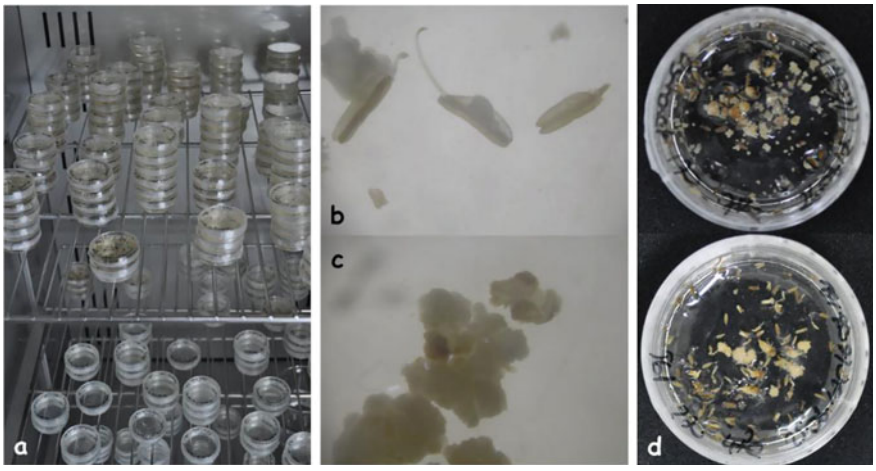

Fig. 3 Androgenic callus formation in induction media. Pictured from inside of phytotron (**a**), image of callus formation from anthers under a binocular microscope (**b**), image of calli under a binocular microscope (**c**), callus formation in induction medium (**d**)

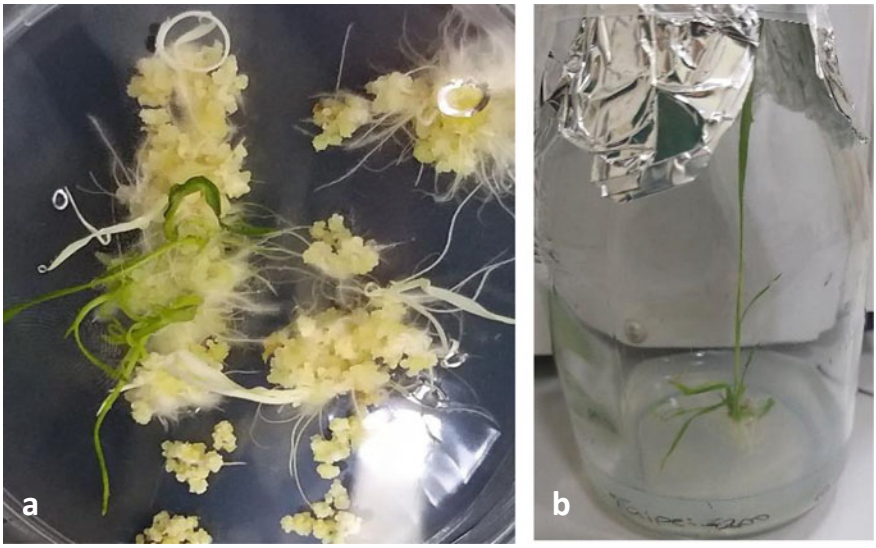

Fig. 4 Healthy plantlets in regeneration medium showing root (**a**) and shoot (**b**) formation

Skoog 1962) containing 1 mg/L 1-naphthaleneacetic acid (NAA), 4 mg/L kinetin, 100 mg/L myo-inositol, 100 mg/L casein hydrolysate, gum arabic, 1 mL/L 1000 × MS vitamin stock solution (Sigma M3900), 30 g/L sucrose as a source of carbon and 6.5 g/L gum agar into media to solidify, pH 5.7–6.0]. Firmly seal the Petri dish with Parafilm M® barrier film. Maintain calli at 27 ± 1 °C, with 12/12 h of light/dark period up to plantlet regeneration (Fig. 4a, b).

Media preparation for seedling regenerations and sterilization. Dissolve 4.3 g/L MS (Murashige and Skoog 1962) basal salt mixture (Sigma M5524) containing 1 mg/L naphthaleneacetic acid (NAA), 4 mg/L kinetin, 100 mg/L myo-inositol and 30 g/L sucrose in 1 L of distilled water at room temperature. Adjust the pH at 5.7–6.0 using 1N NaOH/KOH or 1N HCl. Put 6.5 g gum agar into media to solidify. The media should be autoclaved for 20 min at 121 °C under 1.25 Atm. Put 1 mL/L 1000 × MS vitamin stock solution (Sigma M3900) to the autoclaved medium cooled to 40–50 °C. Store medium preferably at 8–10 °C.

Determination of ploidy level of the regenerants. Chop equal amounts of putative haploid/doubled haploid rice regenerants obtained from anther cultures and tomato (*Solanum lycopersicum* L.) leaf samples, as a standard, together for 30–60 s in 500 μL of ice-cold nucleic extraction buffer. Since the c value of the tomato genome is one of the plants closest to rice, it is often preferred as a standard when analyzing rice in flow cytometry. Add 2 mL of CyStain® PI Absolate P nucleic staining solution (Sysmex 05-5022) on the crude mixture. Pass the suspension through a 30 μm CellTrics® filter (Sysmex) to eliminate cell debris. Collect nuclei into the pre-chilled tubes. Add 2 mL of dye solution previously prepared into the tube. Incubate tubes in the dark

for about 60 min. Analyze the ploidy level of samples by a flow cytometer. We used the Sysmex Partec flow cytometer at Tekirdağ Namık Kemal University, Field Crops Department, Plant Genetics and Cytogenetics Laboratory equipped with a 488 nm Argon laser to excite the PI fluorochrome, and a FL-2 detector with a 585/42 band pass filter. Samples were run on low pressure and 104 nuclei were counted within the double gate (Fig. 5).

Colchicine treatment of haploid plantlets to induce chromosome doubling. Take the haploid seedlings based on the result of the flow cytometer analysis, from regeneration culture (Fig. 6). Wash the roots with tap water and trim back to about 2–3 cm. Immerse trimmed seedlings in 0.2% colchicine with dimethyl sulfoxide (DMSO) and few drops of Tween 20 for 4 h at room temperature. Take treated seedlings out of the colchicine and wash under running tap water overnight. Transplant treated plants into pots containing 2:1:1 soil:sand:peat-moss mixture. Check ploidy of new tillers to confirm chromosome doubling. Doubled haploid tillers will likely have seeds after heading and successful pollination.

Growing the haploid/doubled haloid plantlets. Place the newly transplanted plantlets in a plastic house or growth chamber and cover with plastic bags for a few days for

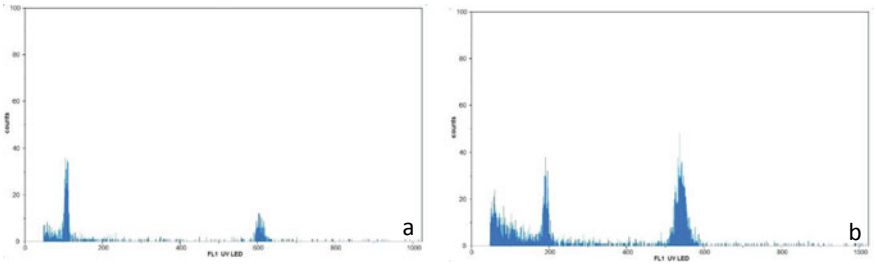

Fig. 5 Ploidy detecting using a flow cytometer. Haploid plantlet (**a**) and diploid plantlet (**b**). First pick related to plantlet obtained from rice anther culture and, for comparison, second pick from a standard plant (*Solanum lycopersicum* L.)

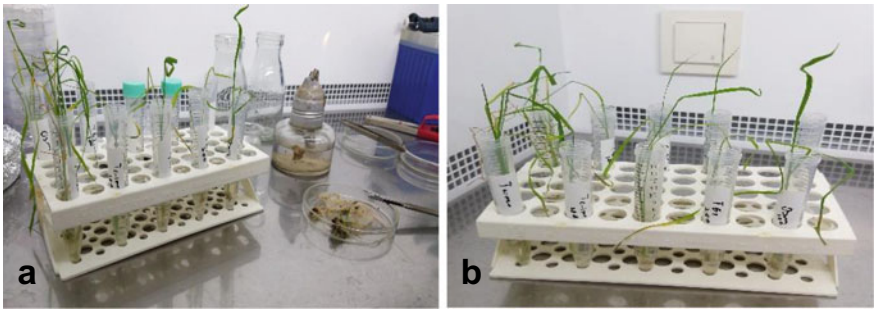

Fig. 6 Colchicine application to rice seedlings found to be haploid as a result of flow cytometer analysis (**a** and **b**)

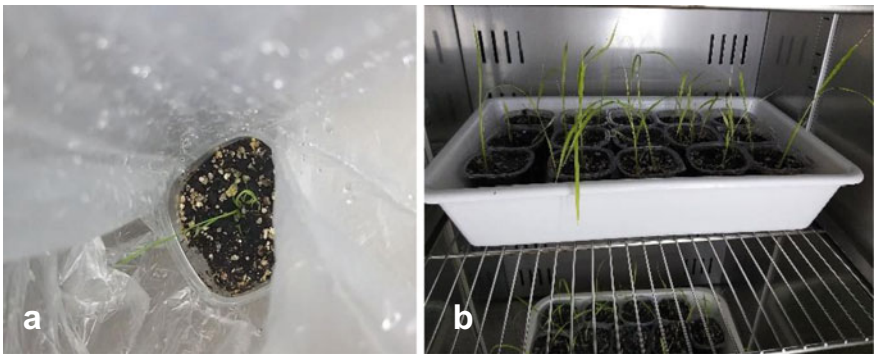

Fig. 7 Plantlet in growth chamber, transferred plantlets into soil for acclimatization (**a**) and plantlets in a growth chamber (**b**)

acclimatization (Fig. 7a). Incubate seedlings at 21/18 °C night/day under 16/8 h of day/night photoperiod (Fig. 7b). Irrigate seedlings with Yoshida's solution (Yoshida et al. 1976) or any other similar nutrient solution.

Validation of the protocols on some rice varieties and M1 population. The optimized protocols were tested on three rice varieties and their M1 populations. M1 seeds were produced by irradiation with 0, 100, 200 and 300 Gy doses of gamma rays. As can be seen from Table 1, an equal number of anthers for each population were planted in the culture medium. In all three cultivars, a decreasing trend is observed in the callus induction percentage, except for the population of Osmancık 97 irradiated with 200 Gy, with increasing radiation dose. It was determined that the percentage of callus induction rate ranged from 17.9 to 19.8% in Taipei 309, 16.2 to 17.3% in Osmancık 97, and 14.4 to 16.6% in TG1. Generally, regenerability of androgenic calli decreased with increasing radiation dosage. In all three cultivars, only albino plants were obtained at the highest radiation dose of 300 Gy. The percentage of green plants obtained in the study also decreases with increasing radiation dose in all three cultivars, except for the 200 Gy gamma rays irradiated population of Taipei 309. The percentage of green plants regenerated in cultures established from the populations obtained by 100 Gy gamma rays doses was 27.3% in Taipei 309 and 25% in both Osmancık 97 and TG1. In cultures established from mutant populations obtained with 200 Gy, green plants regenerated at a rate of 56.7% in Taipei 309, 16.7% in Osmancık 97 and 20% in TG1. TG1 seems the most sensitive rice variety to gamma radiation both in the callus induction stage and plant regeneration stage.

Table 1 Effect of gamma irradiation dose on anther culture ability of rice cultivars

Cultivar	Radiation dose (Gy)	Anther number	No. calli	Callus induction rate (%)[a]	No. regenerated plants[b]	Rate of green plant regeneration (%)[c]
Taipei 309	0	1250	247	19.8	54 (26)*	48.2
	100	1250	240	19.2	44 (12)	27.3
	200	1200	219	18.2	30 (17)	56.7
	300	1100	197	17.9	12	0
Osmancık97	0	1300	223	17.2	40 (14)	35.0
	100	1280	218	17.0	32 (8)	25.0
	200	1200	208	17.3	24 (4)	16.7
	300	1150	186	16.1	10	0
TG1	0	1280	213	16.6	40 (12)	30.0
	100	1250	204	16.3	32 (8)	25.0
	200	1200	173	14.4	20 (4)	20.0
	300	1100	158	14.4	12	0

[a]Callus initiation rate = no. initiated calli/no. incubated anthers × 100%
[b]No. regenerated plants = total no. regenerated plants including albinos, numbers in parentheses are green plantlets
[c]Rate of plant regeneration = no. regenerated green plantlets from callus/no. incubated calli for regeneration × 100%
* Mean comparisons results from LSD test in 5% probability level for callus induction and number of regenerated plantlets and regenerated green plantlets percentage in anther culture of rice

Conclusions

This study was conducted to investigate the potential of obtaining haploid/doubled haploid plants from M1 populations derived from rice seeds irradiated with different doses of gamma radiation. This reflects the potential of introducing novel germplasm resources with new genetic background that can be used for different purposes in breeding programs, such as developing new plant variants resistant/tolerant to *Striga* spp. Regenerability of androgenic callus is particularly sensitive to gamma rays. One-third of the green plantlets obtained were determined as haploid by flow cytometry. We also found that with applying cold pretreatment times over ten days, gamma ray application reduced the rate of obtained healthy plants and increased the rate of obtaining albino plants. As a result of the radio-sensitivity analysis, the GR_{50} value was determined between 250–400 Gy (Brunner 1995; see Chapter "Physical Mutagenesis in Cereal Crops"), depending on the source of gamma radiation and the rice variety. Considering these values, the radiation doses of haploid/doubled haploid rice plants obtained in this study approximately correspond to the GR_{30} and GR_{40} values. Progress in haploid/doubled haploid production, such as the rapid generation of large microspore-derived haploid populations via androgenesis, may be exploited

in mutation breeding. For example, genome-fixed novel mutant variants obtained by anther culture from the irradiated M1 population can be rapidly incorporated into breeding/selection programs to develop novel variants resistant to *Striga* infestation.

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The opinions expressed in this chapter are those of the author(s) and do not necessarily reflect the views of the IAEA: International Atomic Energy Agency, its Board of Directors, or the countries they represent.

Appendix

List of research and technical contract holders, and the secretary of the CRP.

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Japan	Mr. Ken Shirasu RIKEN
Japan	Ms. Satoko Nishimura NARA Institute of Science and Technology (NAIST)
Kenya	Ms. Cathrine Ziyomo Biosciences Eastern and Central Africa (BecA) Hub
Madagascar	Ms. Noronirina Rakotoarisoa Faculty of Sciences, University of Antananarivo
The Netherlands	Mr. Harro J. Bouwmeester Swammerdam Institute for Life Sciences, University of Amsterdam
Sudan	Mr. Ayman Abdel Maged Awad Agricultural Research Corporation (ARC), Biotechnology and Biosafety Research Center
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USA	Mr. Michael Paul Timko Department of Biology, University of Virginia

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