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Introgression of disease resistance genes from *Arachis kempff-mercadoi* into cultivated groundnut

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Abstract

Arachis kempff-mercadoi is a wild species from the section *Arachis*. All *kempff-mercadoi* accessions originate from the Santa Cruz province of Bolivia and they represent *Arachis* species with the A genome. From molecular analysis it was found that although cultivated *A. hypogaea* is made up of A and B genomes, *A. kempff-mercadoi* may not be as closely related to it as are some of the other A genome species. *Arachis kempff-mercadoi* is of interest because it has multiple disease resistance. It was crossed with a Spanish *A. hypogaea* cultivar which is susceptible to foliar diseases and to the insect pest *Spodoptera litura*. The success rate of the cross *A. hypogaea* ($2n = 40$) × *A. kempff-mercadoi* ($2n = 20$) was very low, but it could be increased by culturing immature seeds *in vitro*. Although the hybrids were triploids, a few fertile pollen grains were obtained due to the formation of restitution nuclei in the F_1 plants. Interspecific derivatives at the BC_2F_2 generation were scored for early leaf spot, late leaf spot and to *Spodoptera* damage. Screening results showed that 29% of the derivatives had both early and late leaf spot resistance and that less than 5% of the derivatives had resistance to both the foliar diseases and to *Spodoptera*.

Key words: *Arachis hypogaea* — *Arachis kempff-mercadoi* — *Cercospora arachidicola* — *Phaeoisariopsis personata* — *Spodoptera litura* — interspecific hybrids

The genus *Arachis*, a native of the Brazil-Paraguay region of South America (Simpson et al. 2001), is made up of nine sections (Krapovickas and Gregory 1994). The cultivated species *Arachis hypogaea* L. belongs to the section *Arachis*. Many wild species from the section *Arachis* such as *A. villosa*, *A. correntina*, *A. diogoi* (=*A. chacoense*), *A. stenosperma*, *A. cardenasii*, *A. duranensis* and *A. batizocoi* have been successfully crossed with cultivated species (Stalker 1985, Singh 1986, Stalker and Simpson 1995) with pod formation ranging from 0 to 30%. It is not known if all the pods were mature and the seeds germinated under *in vivo* conditions. Transfer of rust resistance to the cultivated species has been reported from *A. duranensis* (Singh 1986). Simpson et al. (1993) reported the successful transfer of nematode resistance from *A. cardenasii* and *A. diogoi*. Stalker et al. (2002) reported a leaf spot-resistant population from the cross *A. hypogaea* × *A. cardenasii*. The population also had resistance to root knot nematode and southern corn root worm. Milla (2003) has reported the transfer of tomato spotted wilt virus resistance from *A. cardenasii*.

Arachis kempff-mercadoi ($2n = 20$, PI 468331; ICG 8959; Coll no. 30085) is a perennial species and a native of the Santa Cruz province in Bolivia, South America. It belongs to the section *Arachis* with the A genome (Milla 2003). Based on molecular and cytogenetic analysis, *A. hypogaea* is made up of A and B genomes (Singh and Moss 1984, Gimenes et al. 2002). Based on RAPD-molecular analysis it was found that *A. kempff-mercadoi* may not be as closely related to *A. hypogaea* as some of the other A genome species (Mallikarjuna et al. 2003). Although *A. kempff-mercadoi* has been used by Singh (1988) in the crossing programme, there is no published report of transfer of disease resistance and the nature of seed set in the crosses using *A. kempff-mercadoi*.

Surveys at ICRISAT have shown that *A. kempff-mercadoi* has resistance to foliar diseases (Subrahmanyam and Moss 1983, Subrahmanyam et al. 1985, Pande and Narayana Rao 2001) and to the insect pest, *Spodoptera litura* (Stevenson et al. 1993). Although wild species from the section *Arachis* have been recognized by Stalker and Simpson 1995) as cross-compatible with cultivated groundnut (*A. hypogaea* L.), not all wild species from this section cross readily with *A. hypogaea* (Ozias-Akins et al. 1992). The success rate of the cross *A. hypogaea* × *A. kempff-mercadoi* is very low. Very few mature seeds (2%) are produced and a large number of them contain well-developed embryos that have still not reached maturity. These seeds do not germinate *in vivo* but can do so *in vitro*. A small number of pods also had small aborted seeds (3–4 mm). To obtain hybrid plants from aborted seeds, embryo rescue techniques have had to be used (Mallikarjuna and Sastri 1985).

Early leaf spot (ELS) caused by *Cercospora arachidicola* Hori and larly leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk and MA Curtis) are economically significant and widely distributed diseases of groundnut (Waliyar 1991). The incidence and severity of the diseases vary with location, year and cultivar and both diseases cause severe yield losses. Although management options are available, they may not be the best options because of the high costs of fungicides, the possibility of obtaining fungicide-resistant strains and environmental degradation due to the use of chemicals. Hence, the best option to combat these diseases is to obtain disease-resistant cultivars.

The tobacco caterpillar, *Spodoptera litura* (Fab.) is a polyphagous noctuid moth of high reproductive capacity, with the ability to migrate over long distances. These characteristics have resulted in its becoming a pest of many agricultural crops throughout South Asia and South East Asia. Groundnut yield losses up to 71% have been reported in India (Amin 1988). High yield losses of groundnuts have been directly associated with increased larval density of *S. litura*, and the intensity of defoliation (Panchbhavi and Nethradani Raj 1987). With the

development of insecticide resistance to the major chemical groups, including synthetic pyrethroids, the focus has shifted to exploit the genetic diversity of wild *Arachis* species for *S. litura* resistance. The development of groundnut varieties with resistance to *S. litura* has been initiated as an important aspect of integrated pest management (Wightman and Amin 1988).

The objective of this work was to cross the wild species *A. kempff-mercadoi*, which has multiple disease resistance, with *A. hypogaea* and to transfer disease resistance, and thus broaden the genetic base of the crop.

Materials and Methods

Arachis kempff-mercadoi (accession ICG 8959; Coll no. 30085; PI 468331) a wild species from the section *Arachis*, was obtained from the Genetic Resources Unit, ICRISAT, India and an *A. hypogaea* cultivar ICGS 44, a Spanish type cultivar released from ICRISAT, were maintained in a glasshouse. Pollinations were carried out in the morning before 10.00 AM, followed by gibberellic acid (GA; 87.5 mg/l) application to the base of pollinated pistils. Pods were harvested 30–40 days after pollination, or were left on the plant to mature (50–60 days). For embryo germination, pods were thoroughly washed in tap water and sterilized in commercial bleach (Clorox). After repeated washes in sterilized distilled water, the embryos were dissected out of the seeds and cultured on MS (Murashige and Skoog's) basal medium with sucrose (2%), agar (0.7%), NAA (0.1 mg/l) and BAP (1.0 mg/l). Cultures were incubated in the dark at 26°C for the first 7 days. They were later incubated with a 16-h light and an 8-h dark photoperiod at 26°C. After 4 weeks of culture, seedlings with well-developed shoot systems and long taproots were transferred to the rooting medium to induce secondary roots. The rooting medium consisted of 1/10 MS basal medium with NAA (2.0 mg/l) and IBA (1.0 mg/l). Seedlings with robust root systems were transferred to soil. The F_1 hybrid plants were triploids but had some fertile pollen and were selfed. The F_2 plants were used as the female parents and pollinated with *A. hypogaea* pollen. BC_2 plants were scored for ELS, LLS and *S. litura*.

The ploidy of the derivatives was determined by pollen diameter analysis. Three classes of pollen diameter were observed. Diploids had a diameter of 25–27 μM, triploid sterile pollen grains were 25–29 μM, and tetraploids had a diameter of 45–47 μM (Singsit and Ozias-Akins 1992). The pollen diameter of *A. hypogaea* was between 45 and 47 μM and that of *A. kempff-mercadoi* was between 25 and 29 μM. Triploid fertile (2n restitution) grains were 43–45 μM, which was comparable with the pollen grains of tetraploid plants.

Disease screening for ELS and LLS was carried out under simulated conditions by a detached leaf technique (Pande and Narayana Rao 2001). Plastic trays with autoclaved sand were used to place the tetrafoliate leaves in a randomized block design with four replications. The spores for the two fungi were harvested with a cyclone spore collector. The concentration of the inoculum was 20 000 spores/ml. A few drops of surfactant Tween 80 (polyoxyethylene sorbitan mono-oleate) were added. Immediately after inoculation, leaves were placed in a dew chamber at 23°C to ensure wetness of the leaf surface during the night. Plants were removed from the dew chamber the next morning and returned to the glasshouse during the day. The alternating wet and dry period treatments were repeated for 5 days. Plants were then held in the glasshouse until the end of the experiment. The experiment was terminated at the end of 50 days following inoculation. The percentage of defoliation was recorded for ELS and LLS. The leaf area damaged by ELS and LLS was assessed by comparing each leaf with standard diagrams depicting leaves with known percentages of leaf area affected. Disease assessment was scored on a rating scale of 1–100, where a score of 1–10 was rated as highly resistant (HR), 10–20 as resistant (R), 20–50 as moderately resistant (MR) and 50–100 as susceptible (S). Data were collected at 10, 20 and 30 days after inoculation.

Spodoptera litura egg masses were collected on a groundnut crop grown in the experimental block at the ICRISAT research farm. In the laboratory, on hatching, the neonate larvae were reared on a semi-synthetic diet based on chickpea flour and dried sorghum leaves (G. V. Ranga Rao, personal communication, 1999) at 25 ± 2°C and a 14-h light and 10-h dark regime. Three to four pairs of adult moths were released in a cylindrical cage and provided with 10% sucrose solution. The hatched neonate larvae from the egg masses collected from the cages were used. The experiment was replicated with leaflets on each interspecific derivative, which were observed for larval survival and development.

The basal first, third and seventh leaflets of the interspecific derivatives were excised and arranged in a circle in a round plastic pot (4″ diameter), which was kept moist. Ten neonate larvae of *S. litura* were released inside the pot, and sealed with a transparent polythene cover to test their survival on each leaflet. The experimental pots were then transferred to an incubator maintained at 25 ± 2°C, a 14-h light and 10-h dark regime, and with 70% RH. The experiment was replicated with three leaflets per pot of each interspecific derivative. Leaflets were observed for recording survival and development of larvae on each species at 24, 48, 72 and 96 h after release.

Results

Two F_1 hybrid plants were obtained by germination of mature seeds *in vivo* and 11 plants were obtained by germinating well-developed but immature embryos *in vitro*. Hybrid plants from *in vitro* embryo germination and mature seeds from *in vivo* seed germination did not show any morphological difference. Pollen fertility in the F_1 plants ranged from 0 to 5%. F_1 plants were chosen as the female parent and *A. hypogaea* as the pollen donor. In spite of hundreds of pollinations, pod formation was below 10% and pod set was not observed in the reciprocal crosses. Twenty two per cent of the pollinations formed pods, of which 2% of the seeds were mature and the rest were shrivelled seeds. Mature seeds were germinated *in vivo* and BC_1F_2 plants were obtained. The BC_1F_2 plants had 5–7% pollen fertility; again, these hybrid plants were used as the female parents and crossed with *A. hypogaea*. A large number of pollinations induced pegs and many of these pegs later had set pods. Seed set on BC_2F_2 plants was mature and was tetraploids, which was confirmed by pollen diameter analysis of the BC_2F_2 plants.

A total of 105 BC_2F_2 plants were scored for foliar diseases. The results for ELS screening showed that 33% of the plants had resistant reactions to the disease, judging by the leaf tests. There was a direct relationship between defoliation and disease resistance. All the leaf samples that had 0–10% defoliation were highly resistant. Those samples with $< 20\%$ defoliation were in the resistant category. The two categories made up approximately 22% of the derivatives screened. On many of the resistant leaf samples small spots less than 1.00 mm were observed, which did not enlarge to form regular fungal colonies.

Twelve per cent of the BC_2F_2 plants showed resistant reaction to LLS. Five plants did not have any defoliation of the leaflets or disease spots on them. These were highly resistant to LLS. More than half of the derivatives showed 50% or more defoliation with moderately resistant to susceptible reactions to the disease. It was observed that 10% leaf area damage could cause 50–100% defoliation. Here the latent time of leaf area damage was important.

Among 105 plants scored for *S. litura* damage, 65% showed less than 45% neonate mortality. About 30% of derivatives showed moderate to high mortality > 50–69%, with less than 5% of the plants having more than 75% mortality, showing

high levels of antibiosis. A very small percentage of plants showed 100% pest mortality, thus showing highly resistant reactions. The neonate larvae had taken a bite of the leaf nearer to the chewed portion on the adaxial surface of the leaf and regurgitated smears on to the excised leaves. The mortality of larvae on the first, third and seventh leaflets was 25, 31 and 45%, respectively, indicating that feeding on the third leaf conferred a high level of resistance. Even when some of the larvae survived on the leaves, either they did not pupate normally or only abnormal adults were observed.

Among 105 BC_2F_2 plants, one showed highly resistant (zero defoliation with no disease spots) reaction to ELS and LLS. Two derivatives showed a highly resistant reaction to ELS but a moderately resistant reaction to LLS. Three derivatives showed a highly resistant reaction to LLS and a moderately resistant reaction to ELS. About 29% of the derivatives showed different levels of resistance to ELS and LLS. Five derivatives showed resistance to ELS, LLS and *Spodoptera*, of which one derivative was highly resistant to ELS and LLS and moderately resistant to *Spodoptera*.

Discussion

Tremendous progress has been made to improve groundnut as a crop (Holbrook and Stalker 2002). It is well known that groundnut as a crop rests on a narrow genetic base. Isozyme, molecular and pedigree analysis has shown limited diversity in the cultivated species (Grieshammer 1989, Knauft and Gorbet 1989, Kochert et al. 1991). One of the best ways to introduce resistance to biotic constraints (such as foliar diseases and insects) and to introduce genetic variation is by using wild species from the compatible gene pool.

In the present investigation seed set was observed when triploid interspecific hybrids were used as the female parents and crossed with the tetraploid *A. hypogaea*. Triploids obtained as a result of crossing diploid wild species with cultivated *A. hypogaea* have been previously found to be partially fertile (Singh and Moss 1984). The F_1 plants were able to set seeds when used as the female parent but failed to produce seeds when used as the pollen parent. This meant that the discrepancy in the number of chromosomes or loss of a few segments of a chromosome in the egg was tolerated and limited seed set was observed. Although most of the pollen grains were sterile, female fertility was not greatly affected. This was evidenced by formation of 99 seeds as a result of 452 pollinations when F_1 triploids were used as female parents. But only nine of these seeds were filled and well developed.

In earlier investigations, a triploid was treated with colchicine to double the chromosome number (Singh 1986). Hexaploids so obtained were backcrossed to the tetraploid groundnut to obtain mixoploids and, later, tetraploids. This process of obtaining tetraploid derivatives took over 3 years. At every generation, screening for target diseases had to be carried out. This route was time-, labour- and resource-consuming. In the present investigation, tetraploidy was achieved in one step and the time taken was reduced by 1.5 years.

Instead of the conventional method of ploidy determination by chromosome counts, pollen diameter is a good indicator of the ploidy of the hybrid. The presence of 2n gametes obtained as a result of restitution nuclei is common in triploids obtained in crosses involving diploid wild species and the tetraploid cultivated groundnut (Singh and Moss 1984, Singsit and Ozias-Akins 1992).

Crosses with *A. kempf-mercadoi* yielded derivatives resistant to ELS and LLS. Many of these derivatives also showed resistant reactions to rust under simulated conditions. Resistance to *Spodoptera* was successfully transferred from *A. kempff-mercadoi* into the derivatives. Many of the derivatives showed either resistance to ELS or LLS. Combined resistance to both diseases was observed in 30 derivatives with five derivatives showing disease resistance to all three constraints.

The mortality of neonate larvae of *S. litura* on hybrids indicates high levels of resistance (Lynch et al. 1981, Stevenson et al. 1993). Some neonates were also found dead on the stems of the excised leaves due to the hairiness of the plant. The larval mortality on the leaf surface suggests the involvement of chemicals, and poor larval growth on some excised leaf material also contributed to resistance against *S. litura* larvae. Stevenson et al. (1993) noticed high mortality of neonate larvae and their retarded development on excised leaves of wild species of *Arachis*: results similar to those obtained in the present investigation. Similarly, several species of wild *Arachis* had incomplete larval development and a very high larval mortality of *S. frugiperda* (Lynch et al. 1981). Apart from leaf chemistry, the toughness of leaves in the derivatives may have played a role in conferring resistance.

It is evident that the ELS-, LLS- and *Spodoptera*-resistant genes in *A. kempf-mercadoi* are not linked and probably their loci are in different regions or on different chromosomes. Hence, to bring multiple disease resistance genes into derivatives, resistant derivatives may have to be crossed, thus pyramiding the resistance genes can be achieved. The present investigation opens up vistas in groundnut improvement by accessing resistance genes to foliar diseases and *Spodoptera* from *A. kempff-mercadoi* and transfer to cultivated groundnut. The results show that wild species from the section *Arachis* are amenable to gene transfer through wide hybridization.

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