Production of Hybrids Between *Arachis hypogaea* and *A. chiquitana* (section *Procumbentes*)[1]

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ABSTRACT

There is no report of successful crosses between cultivated peanut (*Arachis hypogaea* L.) and wild species from section *Procumbentes*. Interspecific hybrids between *A. hypogaea* (section *Arachis*) and *A. chiquitana* Krapov., W.C. Gregory & C.E. Simpson (section *Procumbentes*) were produced by the application of growth regulators to pollinated pistils, and hybrid plants were obtained for the first time by germinating hybrid embryos *in vitro*. Eleven hybrids were produced, of which one hybrid did not survive to the flowering stage, but the other hybrids were fertile. The hybridity of the F_1 plants was confirmed by SSR analysis and one BC_1 plant gave rise to mature seeds. In addition, *A. chiquitana* has been identified as one of the few wild species of *Arachis* showing resistance to *Aspergillus flavus* colonization. *Aspergillus flavus* produces aflatoxin, a carcinogenic agent causing liver cancer, a serious post harvest constraint to peanut production world wide. Initial seed screening of *A. chiquitana* for *A. flavus* showed promise of obtaining hybrids resistant to *A. flavus* colonization, but it is unknown if the interspecific hybrids would also have aflatoxin resistance. This is the first report of obtaining hybrids between *A. hypogaea* and *A. chiquitana*.

Key Words: Peanut, groundnut, wild species, interspecific hybridization, embryo germination, *Aspergillus flavus*, aflatoxin.

According to the published literature on the crossability of the species in the genus *Arachis*, none of the species outside section *Arachis* will hybridize with the cultivated peanut (*Arachis hypogaea* L.) without the aid of *in vitro* methodology. In many instances when hybrids are produced between *A. hypogaea* and wild species outside the section *Arachis*, hybrids are completely sterile or, in other words, genetic dead-ends (Stalker and Simpson, 1995). With the development of embryo rescue and tissue culture techniques, hybrids between *A. hypogaea* and *A. glabrata* Benth., (section *Rhizomatosae*) have been reported (Mallikarjuna and Sastri 1985, 2002; Shen *et al*., 1995) *Arachis chiquitana* Krapov., W.C Gregory and C.E. Simpson, is a wild species collected from Chiquitos province of Santa Cruz in Bolivia, and although it was previously placed in section *Erectoides* by Gregory and Gregory (1979), it is currently placed in section *Procumbentes* (Krapovickas and Gregory, 1994).

Aflatoxin contamination of peanut, caused by the *Aspergillus flavus* group of fungi, is one of the most important constraints to quality peanut production in the semi-arid rainfed areas of the world. This is particularly significant in relation to public health and international trade (Waliyar *et al.*, 1994). In addition to peanuts, aflatoxins are found in many agricultural crops such as corn, cotton, wheat, and rice and consumption of the toxin by humans can lead to liver cancer. Household and Hunt (1991) also reported a striking association between exposure to aflatoxin in children and both stunting and low weight gain. In West Africa, people are chronically exposed to high levels of aflatoxin starting in the utero and continuing throughout life (Gong *et al*., 2002). Genetic resistance should be one of the major components in a strategy to manage aflatoxin contamination in peanut, but high levels of genetic resistance to aflatoxin have not been identified in cultivated peanuts. Ghewande et al., (1989) reported accessions of *A. duranensis* Krapov. and W.C. Gregory and *A. cardenasii* Krapov. And W.C. Gregory as being highly resistant to *in vitro* seed colonization to *Aspergillus flavus.* Xue et al., (2004) confirmed resistance in three *A. duranensis* and two *A. cardenasii* accessions after testing 36 accessions of the two species. *Arachis chiquitana* is one of the three wild species identified as resistant to *Aspergillus flavus* Link ex. Fries colonization and subsequent aflatoxin production (Thakur *et al*, 2000). Pande and Rao (2001) also identified *A. chiquitana* as one of the wild species resistant to late leaf spot (LLS), a fungal disease caused by *Phaeoisariopsis personata* (Berk. & M.A. Curtis) Deighton. Hence, developing strategies to cross *A. hypogaea* with *A. chiquitana* not only broadens the genetic base of cultivated peanut

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but will also potentially bring in other desirable genes.

Materials and Methods

Seeds of *A. chiquitana* with $2n = 2x = 20$, (ICG 11560; collector number 36025; PI 476004; Fig. 1A) from section *Procumbentes* were obtained from the genetic resources division of ICRISAT and grown in a glasshouse. Seeds of *A. hypogaea* cv. ICGS 44 ($2n = 4x = 40$) were also grown and maintained in the glasshouse. Cross pollinations using *A. hypogaea* as the female parent and *A. chiquitana* as the pollen donor were carried out before 10:00 a.m. Emasculations of anthers from flowers were carried out the previous evening. Application of 75 mg/L of gibberellic acid (GA) at the base of pollinated pistils was mandatory to obtain pods from cross pollinations. A total of 493 pollinations were carried out and 167 pods were harvested between 25 and 30 days after pollination. Pods were then surface sterilized and ovules were extracted under sterile conditions. Ovules which were more than 4.0 to 5.0 mm long were dissected and the embryos (immature seeds) were cultured directly on the semisolid growth medium (Fig. 1E). The growth medium consisted of MS (Murashige and Skoog, 1962) basal medium with 3% sucrose plus napthalene acetic acid (NAA; 0.1 mg/L) and benzylamino purine (BAP; 1.0 mg/L). Ovules less than 4.0 mm in size were not used in the experiment.

Embryos germinated and gave rise to seedlings (Fig. 1F) with individual or multiple shoots. Shoots were rooted *in vitro* on rooting medium, consisting of ½ MS basal salts (plus sucrose), NAA (2.0 mg/L), indoleacetic acid (IAA; 1.0 mg/L). After 15 d on rooting medium, shoots were transferred to ½ MS basal medium (without growth regulators). Healthy roots developed within 3 wk of culture. Shoots with well-developed roots were transferred to sand and acclimatized under controlled conditions at 24 C and RH of 72–75%. A month of acclimatization was sufficient to transfer the plants to a glasshouse.

DNA was extracted from young, folded leaflets with Qiagen miniprep kits (Qiagen, Valencia, CA) and amplified by means of 30 pmol primer, 5 ng template DNA, 4 mM $MgCL_2$, 0.2 mM dNTPs, 1 U Taq polymerase with 1 × reaction buffer in a total reaction volume of 20 µL. Reaction conditions were 94 C for 2 min, 35 cycles of 94 C for 45 sec, empirically defined annealing temperature of 60 C for 1 min, 72 C for 90 sec, then a final extension of 10 min at 72 C. Amplification products were visualized on non-denaturing 9% 29:1 (w/w) polyacrylamide gel followed by silver staining. Silver staining consisted of 3 min in H_2O, 20 min in 0.1% (w/v) CTAB, 15 min in 0.3% ammonium solution, 15 min in a solution of 1 M NaOH, 0.1% silver nitrate and a few drops of 25% ammonium solution, and a rinse in H_2O, and followed by development in a 1.5% NaC solution with 0.02% by volume formaldehyde solution. The SSR procedure and primers are described in detail in Ferguson *et al.* (2004). Amplification products were noted as present or absent. The four SSR primers used to distinguish the parents and the hybrids, were as follows:

1. pPGPseq3F1 Forward primer—AGCGATCAATCGGTTTCAAG, fragment length—290 bp. Reverse primer—GAAACGAAACGAAGACCGAA.
2. pPGPseq4D4 Forward primer-CGGCTGTTAGGTAATCAGTTCA, fragment length-187 bp. Reverse primer-TCAACAGGAATAGCTGCACG.
3. pPGPseq2A6 Forward primer-GCTTCTTCGTTGTTGCCTTC, fragment length-249 bp. Reverse primer-TGCCAGTTGTTCATAGCTTCA.
4. pPGPseq4H11 Forward primer-ATCACCATCAGAACGATCCC, fragment length-269 bp. Reverse primer-TTGTAGCCTTCTGGCGAGT.

The ploidy of the derivatives was determined by pollen diameter analysis. Three classes of pollen diameter were observed. Diploids had a diameter of 25 to 27 µM, triploids were 25 to 29 µM, and tetraploids had a diameter of 45 to 47 µM (Singsit and Ozias-Akins, 1992). Pollen diameter of *A. hypogaea* was between 45 to 47 µM and that of *A. chiquitana* was between 25 to 29 µM. Pollen in triploids which had undergone 2*n* restitution had 43 to 45 µM (and apparently fertile) was comparable to pollen grains of tetraploid plants.

Nineteen seeds were surface sterilized and were uniformly wounded by pricking with a sterile needle to allow the invasion of *A. flavus* spores. Seeds were spray inoculated with *A. flavus* spore suspension (1×10^6 spores/mL). Petri dishes with sprayed seeds were placed in high humidity and incubated at 25 C. Individual seeds were scored for *A. flavus* colonization, using a rating scale of 1 to 4 as described by Thakur *et al.* (2000).

Results

Pods from cross pollinations using *A. chiquitana* as the pollen parent appeared similar to *A. hypogaea* but were mostly single seeded. Thirty-four percent of the pollinations formed pods from

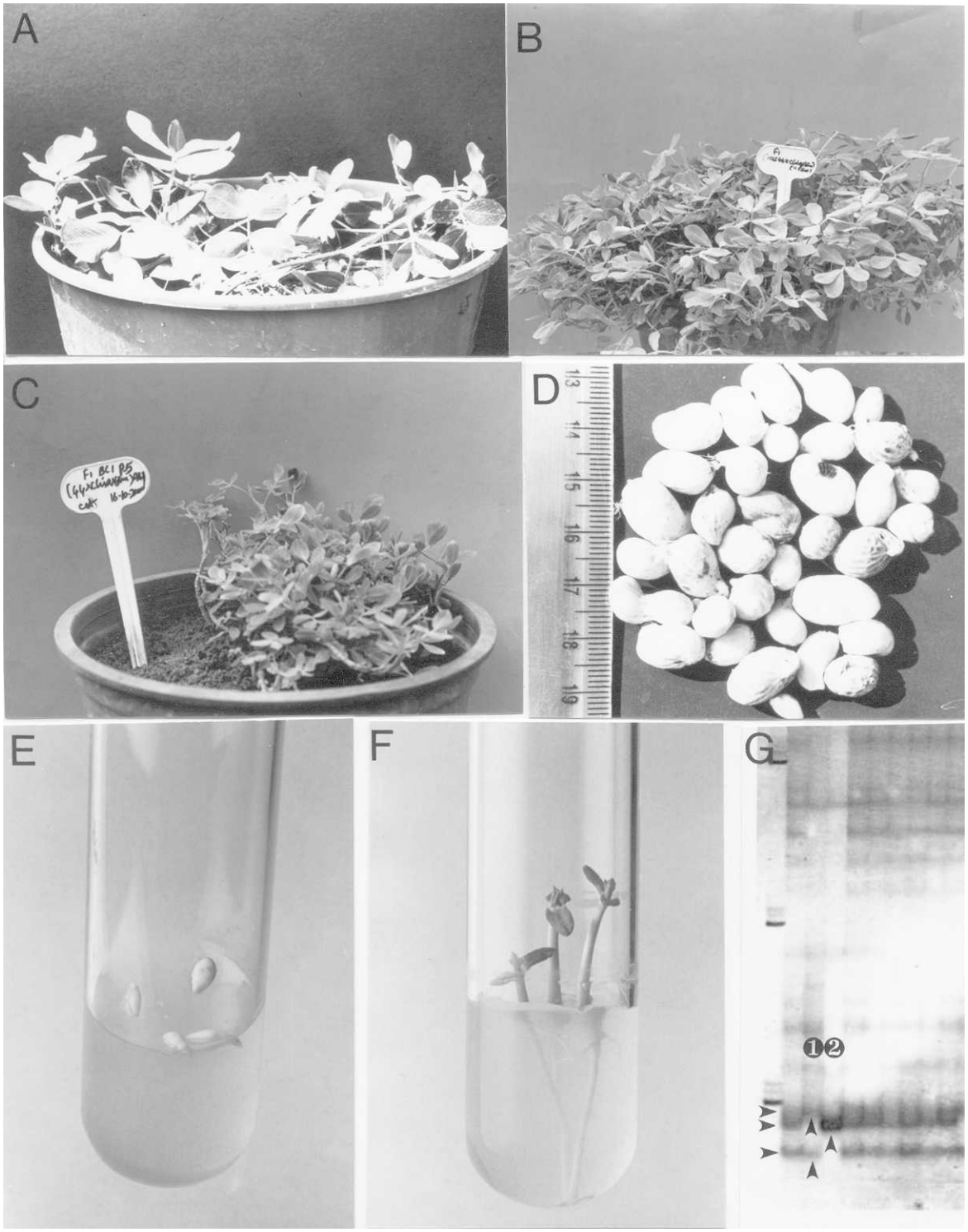

Fig. 1. Crossability between *A. hypogaea* and *A. chiquitana*.
A. Plant of the wild species *A. chiquitana*.
B. F_1 hybrid plant from the cross *A. hypogaea* × *A. chiquitana*.
C. BC_1 hybrid plant of the cross *A. hypogaea* × *A. chiquitana*.
D. BC_2 pods showing mostly single seeded and a few double seeded pods.
E. Large but immature embryos from the cross *A. hypogaea* × *A. chiquitana* on germination medium.
F. Germinating seedlings from the cross *A. hypogaea* × *A. chiquitana*.
G. SSR-3F1, fragment length 290 base pairs: analysis of the parents and the hybrids. From left to right: 1st lane: Molecular weight marker; 2nd lane: F_1 hybrid, hybrid bands have been marked with arrows; 3rd lane: *Arachis hypogaea*, the female parent, marked no. 1; 4th lane: *A. chiquitana*, the male parent, marked no. 2; Lanes 5 to 10: F_1 hybrids, hybrid bands have been marked with arrows.

the cross *A. hypogaea* × *A. chiquitana*. Aborted and dried seeds were obtained if harvested beyond 30 d. Forty-seven percent of the ovules (immature seeds) were large (>4.0 × 2.5 mm), rest of the seeds less than 4.0 mm long were discarded. A total of 25 large ovules were dissected and embryos were cultured (Fig. 1E). Approximately 30 d was required for the embryos to respond to culture and develop into healthy seedlings (Fig. 1F) or form multiple shoots. If the hybrid pods with large ovules were allowed to dry, then they did not germinate *in vivo* or *in vitro*. Eleven hybrid plants were obtained (Fig. 1B). One plant did not reach the flowering stage, in spite of having normal vegetative growth. The hybrid plants had intermediate morphology with the leaf shape resembling that of *A. chiquitana*. All the 10 plants flowered profusely and pollen fertility ranged from 5 to 24%.

Hybrid plants were backcrossed using *A. hypogaea* as the pollen parent. It was possible to obtain mature BC_1 pods from one hybrid plant, whereas the remaining plants did not set mature seeds. BC_1 pods were mostly single seeded and abnormal in their shape. Twelve BC_1 hybrid plants were obtained (Fig. 1C), from which two remained in the juvenile stage with little vegetative growth. Four of the plants had good vegetative growth but did not reach the flowering stage. Six hybrid plants flowered after propagation for at least 180 d. Pollen fertility in the BC_1 hybrids ranged from 18 to 24%, and were tetraploids ($2n = 4x = 40$) which was confirmed by pollen diameter analysis. Mature BC_2 seeds were obtained, but in small number, ranging from one to 11 seeds. Most of the pods were single seeded, and the few double seeded pods produced severe constriction between the seeds (Fig. 1D).

Four SSR markers showed polymorphism between the two parents. SSR 3F1 (Fig. 1G) showed heterozygosity between the two parents. Two unique bands were consistently seen in *A. hypogaea*, whereas *A. chiquitana* had one distinct band. All seven F_1 hybrids used in the study showed the presence of bands from both the parents.

To check if it was possible to transfer resistance to *A. flavus* colonization, seeds were subjected to *A. flavus* colonization following the method described by Thakur *et al.* (2000). Large variation was observed with respect to fungal colonization. Individual seeds were scored for surface colonization, and the results showed six seeds which had the rating of 1 (resistant), and 12 seeds had the rating of 4 (susceptible). The other seeds showed a rating of 2 or 3. Since the method to test for the presence of aflatoxin is destructive to the seed (Devi *et al.*, 2000), seeds were not sacrificed to test for the presence of aflatoxin. Once large numbers of seeds are produced, the logical next step will be to test seeds for *A. flavus* colonization and aflatoxin production.

Discussion

Wild species from section *Arachis* have been used in the improvement of peanut because they are compatible with cultivated peanut (Mallikarjuna *et al.*, 2004). Although there have been various attempts in the past to cross wild species from other sections of *Arachis*, the attempts have not been successful because of barriers to crossability. Even if the barriers are overcome by the use of various *in vivo* or *in vitro* techniques, many times the hybrid plants are male and female sterile or they do not mature into the reproductive phase (Stalker and Simpson, 1995). Tissue culture techniques first developed to recover aborting embryos from crosses between *A. hypogaea* and *A. glabrata* (Mallikarjuna and Sastri, 2002), a wild species belonging to section *Rhizomatosae*, can be applied to recover hybrid embryos from other intersectional crosses (Mallikarjuna, 2002) as is evident from the present study. Crosses between *A. hypogaea* and *A. paraguariensis* (ICG 8130; PI 337350 KCF 11462) and *A. hypogaea* and *A. appressipila* (ICG8945; PI 468149; GK 30003) were obtained by the use of tissue culture techniques. The hybrids had profuse vegetative growth but did not reach reproductive stage (Mallikarjuna, unpubl. data), and were considered genetic dead-ends. The hybrids obtained in the present study as well as with other *A. hypogaea* intersectional hybrids indicate that a generalized statement cannot be made that all hybrids involving wild species outside section *Arachis* are genetic dead-ends.

It would be of interest to pursue crosses involving *A. chiquitana* because it is one of the few wild species that can be crossed with *A. hypogaea* to potentially transfer resistance to *A. flavus*. The other species that have resistance to *A. flavus* are *A. pusilla* and *A. triseminata* (Thakur *et al.,* 2000), *A. duranensis* and *A. cardenesaii* (Xue *et. al.*, 2004), the latter two species are cross compatible with *A. hypogaea*.

The present study shows that the crossability barriers are not rigid and with the manipulation of embryo rescue techniques available for peanut, many more species from different sections may be successfully crossed with cultivated peanut.

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