*Euphytica* **126:** 161–167, 2002.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*


# Morphological, cytological and disease resistance studies of the intersectional hybrid between *Arachis hypogaea* L. and *A. glabrata* Benth.

Nalini Mallikarjuna & D.C. Sastri
*International Crops Research Institute for Semi-Arid Tropics, Patancheru P.O. 502 324, Andhra Pradesh, India*

Received 7 May 1999; accepted 5 March 2001

*Key words: Arachis glabrata*, cytology, disease resistance, electrophoresis, groundnut, interspecific hybridization

## Summary

*Arachis glabrata* Benth, variety *glabrata* collector GK 10596 (PI 276233; ICG 8176) belonging to section *Rhizomatosae* has resistance to rust, late leaf spot and viral diseases. *A. hypogaea* L. cv MK 374 (section *Arachis*) is susceptible to rust, late leaf spot and to the viral diseases peanut stripe, peanut mottle and peanut bud necrosis. Hybrids between *A. hypogaea* cv MK 374 and *A. glabrata* were produced after interspecific pollinations and embryo culture. The hybrids produced had morphological characters of both parents plus floral abnormalities not seen in either parent. It was possible to identify the hybrids by esterase isozyme analysis when still in culture. Cytological research showed variable chromosome association and also homeology between the genomes of *A. hypogaea* and *A. glabrata*. The hybrids inherited resistance to rust, late leaf spot, peanut bud necrosis and peanut stripe diseases from the pollen parent *A. glabrata*.

## Introduction

Wild species have proven their importance as contributors not only to the evolution of crop taxa but also as donors of several desirable genes for genetic improvement of crop plants. The wild species *Arachis glabrata* Benth., the only tetraploid wild species outside section *Arachis* and which belongs to section *Rhizomatosae,* shows resistance to fungal, viral and insect pests. Although *A. glabrata* has the same chromosome number as cultivated groundnut (*A. hypogaea* L.), it is cross incompatible with cultivated groundnut because of hybridization barriers.

Techniques are now available to cross *A. hypogaea* with *A. glabrata* (Sastri & Moss, 1982; Mallikarjuna & Sastri, 1985 a & b). The present paper describes morphological traits, meiotic behavior, isozyme analysis and reaction to rust, late leaf spot (LLS), peanut bud necrosis (PBNV), peanut mottle virus (PMV), and peanut stripe virus (PSTV) of the hybrid between *A. hypogaea* cv MK 374 and *A. glabrata*.

## Materials and methods

*A. hypogaea* cv MK374 was used as the female parent and crossed with *A. glabrata* variety *glabrata*: GK-10596 (PI 276233; ICG 8176). Emasculations, pollinations and growth regulator applications were carried out as described by Sastri & Moss (1982). Pods were harvested between 40–50 days after pollination. Pods containing immature ovules were cultured as described by Mallikarjuna & Sastri (1985b).

Hybrid shoots (*A. hypogaea* × *A. glabrata*), which were 4–6 cm long, were transferred to the rooting medium. The rooting medium consisted of 1/10 MS (Murashige & Skoog's medium) basal salts with 3% sucrose and with 2.0 mg/l naphthalene acetic acid (NAA) and 1.0 mg/l indole butyric acid (IBA). After 15 days, the shoots were transferred to 1/10 MS basal medium. Fifty eight percent of the shoots rooted. Rooted shoots were transferred to sand and soil mixture (1:1) and were acclimatized in an incubator at 25 °C, with 80% relative humidity. Established plants were initially maintained in the glasshouse. They were later transferred to big cement rings to encourage their perennial growth habit and were maintained under

field conditions. It was possible to maintain hybrid shoots *in vitro* indefinitely. Hybrid shoots were rooted *in vitro* whenever desired and transferred to soil. Variation in somaclones was not observed. Six hybrid clones (plants) were selected for analysis.

For meiotic analysis, suitable flower buds from the $F_1$ hybrid was fixed in Carnoy's II fluid (aceticacid 1: chloroform 3: and ethanol 6). For mitotic study, healthy immature leaflets 2–3 mm in size were treated in a saturated solution of α-bromonapthalene in distilled water for three hours and fixed in Carnoy's I (acetic acid 1: ethanol 3). To obtain mitotic plates, leaftips were squashed in 2% acetocarmine. To analyze the pollen grains for fertility, flowers were collected in the morning on the day of anthesis. Individual elongated anthers from the flowers were squashed in 2% acetocarmine, gently warmed and were scanned after 3 hr when stainable and non-stainable grains were counted.

For localizing esterases, the method of Scandalios (1969) was followed. Proteins were extracted from immature leaves from potted plants, in 0.1 m tris buffer (pH 6.8) and polyacrylamide gel electrophoresis was carried out on 7% slab gels. Hybrid plants were screened for rust and late leaf spot diseases by the detached leaf technique as described by Subrahmanyam et al. (1982).

The screening method for the three virus diseases viz. PBNV, PMV and PSTV was by mechanical sap inoculation. Plant extracts containing the viruses in an appropriate buffer were applied to the surface of the leaves of healthy-looking hybrid plants. Disease-infected seeds of *A. hypogaea* cv TMV 2 were used as positive controls and healthy uninfected seeds of the same cultivar were used as negative controls. The presence of virus was tested by the ELISA method as described by Hobbs et al. (1987). Absorbence values at 620 nm were determined with a Titertek Multiscan ELISA reader. ELISA readings above 1.0 were considered as positive for the presence of virus.

## Results

Fifteen different plants which were individual hybrids between *A. hypogaea* and *A. glabrata* were successfully established in soil. The hybrid plants showed steady growth. Not all the hybrid plants reached the flowering stage. Only one hybrid plant showed steady growth for four months before flowering in the winter months (15–16 °C min and 25 °C max) unlike the parents which had few to no flowers in winter.

*Table 1.* Variation in flower morphology

| Observation | # (%) |
|---|---|
| Normal with 5 papilionaceous petals | 360 (29) |
| Lacking left wing petal | 277 (23) |
| Lacking right wing petal | 393 (32) |
| Lacking both the wing petals | 101 (8) |
| Lacking the standard petal | 34 (3) |
| More than one standard | 10 (1) |
| Abnormal with short hypanthium | 52 (4) |
| Total | 1227 |

*A. hypogaea* is an annual plant with a life span of approximately 130 days, while *A. glabrata* is a perennial with underground rhizomes. The hybrids were perennial, but lacked rhizomes. Leaflets in *A. hypogaea* were obovate and soft in texture, whereas in *A. glabrata* they were lanceolate and coriaceous. The F1 hybrids showed unifoliate, bifoliate, tri- and tetrafoliate leaves but later stabilized to four leaflets, as seen in both parents, but the leaves were lanceolate and coriaceous as in *A. glabrata*.

Flower structure and length, and the colour and size of the standard petal were variable. Some of the flowers looked like those of *A. hypogaea* cv MK374 with 2–3 cm long hypanthium. The standard petal was orange in colour with dark orange markings. Some flowers resembled *A. glabrata* with hypanthium longer than 4 cm and with no dark orange markings on the standard petal (Figure 1d). The variation was not specific to any branch. Variation was also observed with respect to the structure of the flower. Most often one of the wing petals was missing (Figure 1e & f; Table 1). Sometimes there was an additional standard petal (Figure 1g), which was either complete or incomplete. There were even a few flowers (< 1%) which lacked the keel petals.

Esterase isozyme profiles of the $F_1$ hybrid plants compared to those of the parents showed bands specific to *A. glabrata* and *A. hypogaea* cv MK 374 (Figure 1a). It was possible to identify the $F_1$ hybrid plants at a very early stage even before the hybrid shoots were rooted *in vitro*.

Leaftip squashes of the hybrid showed the presence of 40 chromosomes. Twenty cells were analyzed and all the cells showed the presence of one small single chromosome (Figure 2e), characteristic of the

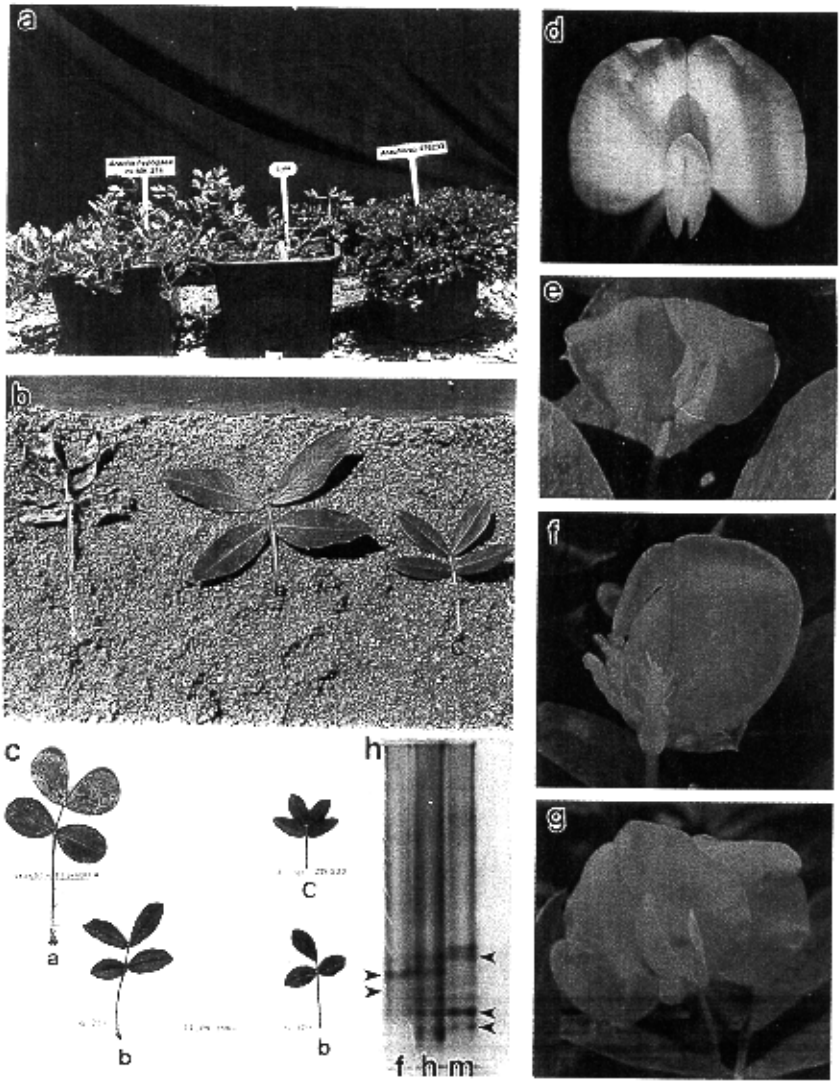


*Figure 1.* Intersectional hybrid in *Arachis*. a. Female parent *A. hypogaea* cv MK 374; hybrid plant; *A. glabrata*, the male parent. b. Late leaf spot disease reaction seen on the parents and the hybrid. Note that the hybrid (b) and the male parent (c) are showing resistant reaction, whereas the female parent (a) has withered under disease pressure. c. No rust disease reaction seen on the male parent (c) and the hybrid plants (b), whereas the female parent (a) shows susceptible reaction. d. Normal groundnut flower. e. Hybrid flower with right wing petal missing. f. Hybrid flower with left wing petal missing. g. Hybrid flower with additional standard petal. h. Esterase isozyme band pattern of the parents (f & m) and the hybrid (h). Arrows point at diagnostic bands.

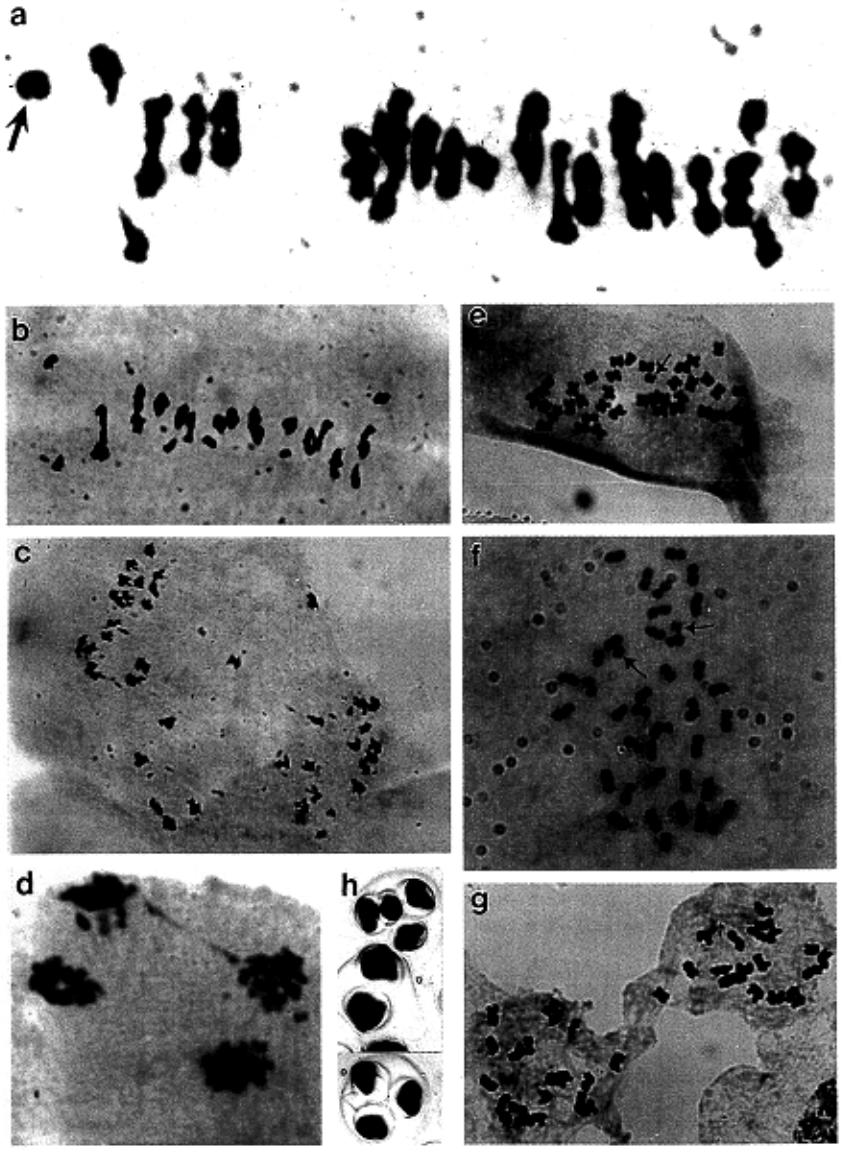


*Figure* 2. Cytological analysis of the parents and the hybrid. a. Meiotic metaphase of the hybrid showing 19 bivalents and 2 univalents. The arrow points at the small chromosome which has not paired. (× 6225) b. Metaphase of the hybrid showing univalents, bivalents, trivalent and tetravalent. (× 1500) c. Anaphase I of the hybrid showing 7 laggards. (× 1600) d. Anaphase II of the hybrid showing a bridge. (× 1800) e. Mitotic metaphase of the hybrid. Arrow points at the single small chromosome. (× 1200) f. Mitotic metaphase of *A. hypogaea*. Arrows point at two small chromosomes. (× 1500) g. Mitotic metaphase of *A. glabrata*. Note the absence of small chromosomes. (× 1500) h. Diads, triads and tetrads of the hybrid. (× 450)

'A' genome and a contribution of *A. hypogaea*. Root tip squash of *A. hypogaea* showed two such small chromosomes (Figure 2f) which were absent in *A. glabrata* (Figure 2g).

*A. hypogaea* had normal meiotic pairing with 20 ring bivalents in 96% of the meiocytes analysed. In *A. glabrata* a mean of 2.3 quadrivalents, 1.13 univalents and 0.2 trivalents per pollen mother cell were observed in the 35 pmcs (pollen mother cells) observed.

Chromosome associations in the hybrid were highly variable (Table 2). Formation of twenty bivalents was very rare and was seen in only one pmc. The number of bivalents ranged from 9–19 (Figure 2a). The number of ring bivalents (mean 9.8 per pmc) and rod bivalents (mean 5.52 per pmc) also varied from 6–13 and 1–11, respectively. Eleven ring bivalents and 6 rod bivalents were most frequent and were observed in 19% of the pmcs. Univalents (mean 5.0 per pmc) ranged from 0–12 per pmc, with four univalents being present in 20% of the pmcs. At least one trivalent was observed in 43% of the pmcs and a tetravalent in 46% (Figure 2b). Average numbers of trivalents and tetravalents per pmc were 0.35 and 0.39 respectively. Three pmcs showed one pentavalent each.

Bridges and laggards were observed at anaphase I and II (Figure 2 c &d). In 20% of the cells, 1–3 laggards (chromosomes) were found and bridges ranging from 1–3 were seen in 19% of the cells at anaphase I. At anaphase II, 28% of the cells showed 1–4 laggards and 30% of the cells showed 1–3 bridges (Table 3). At the tetrad stage, a few diads and triads were observed (Figure 2h).

Anthers were thin and remained indehiscent even after the flower opened. The proportion of pollen grains stainable with acetocarmine varied. Pollen stainability ranged from 4–26% and variation in stainability was observed among anthers of a flower and also between flowers. Analysis of variance indicated that there were no significant differences between flowers or within a flower.

Screening tests for the presence of peanut bud necrosis virus (PBNV) by aphid inoculation did not show disease symptoms, although insect feeding spots were located. This meant that although initially the insect tried to feed on the leaves it was not successful in transmitting the virus, and hence the disease. Similarly hybrid plants inoculated with PSTV virus showed no disease symptoms. ELISA tests also showed negative readings of 0.07 and 0.08, the same as that of negative control, for the presence of PBNV and PSTV in the

*Table 2.* Chromosome configurations in the hybrid plant (*A. hypogaea* × *A. glabrata*)

| Chromosome association | | | | | No. of cells |
|---|---|---|---|---|---|
| II | I | III | IV | V | (%) |
| 20 | 0 | 0 | 0 | 0 | 1(1) |
| 19 | 2 | 0 | 0 | 0 | 5(7) |
| 18 | 4 | 0 | 0 | 0 | 7(9) |
| 18 | 1 | 1 | 0 | 0 | 3(4) |
| 17 | 6 | 0 | 0 | 0 | 4(5) |
| 17 | 2 | 0 | 1 | 0 | 1(1) |
| 17 | 3 | 1 | 0 | 0 | 7(9) |
| 17 | 2 | 0 | 1 | 0 | 3(4) |
| 16 | 5 | 1 | 0 | 0 | 2(3) |
| 16 | 4 | 0 | 1 | 0 | 5(7) |
| 16 | 2 | 2 | 0 | 0 | 1(1) |
| 16 | 1 | 1 | 1 | 0 | 1(1) |
| 15 | 10 | 0 | 0 | 0 | 1(1) |
| 15 | 7 | 1 | 0 | 0 | 1(1) |
| 15 | 6 | 0 | 1 | 0 | 5(7) |
| 15 | 5 | 0 | 0 | 1 | 1(1) |
| 15 | 3 | 1 | 1 | 0 | 2(3) |
| 15 | 2 | 0 | 2 | 0 | 1(1) |
| 14 | 12 | 0 | 0 | 0 | 1(1) |
| 14 | 9 | 1 | 0 | 0 | 1(1) |
| 14 | 8 | 0 | 1 | 0 | 1(1) |
| 14 | 5 | 1 | 1 | 0 | 1(1) |
| 14 | 4 | 0 | 2 | 0 | 1(1) |
| 14 | 4 | 1 | 0 | 1 | 1(1) |
| 13 | 8 | 2 | 0 | 0 | 1(1) |
| 13 | 7 | 1 | 1 | 0 | 4(6) |
| 13 | 4 | 2 | 1 | 0 | 1(1) |
| 12 | 10 | 2 | 1 | 0 | 1(1) |
| 12 | 9 | 1 | 1 | 0 | 1(1) |
| 12 | 8 | 0 | 2 | 0 | 1(1) |
| 12 | 7 | 0 | 1 | 1 | 1(1) |
| 12 | 6 | 2 | 1 | 0 | 2(3) |
| 12 | 5 | 1 | 2 | 0 | 1(1) |
| 11 | 11 | 1 | 1 | 0 | 1(1) |
| 11 | 10 | 0 | 2 | 0 | 1(1) |
| 11 | 7 | 1 | 2 | 0 | 1(1) |
| 9 | 8 | 2 | 2 | 0 | 1(1) |

*Table 3.* Chromosome distribution at anaphase I and II in the hybrid *A. hypogaea* × *A. glabrata*

| | Anaphase I | Anaphase II |
|---|---|---|
| Total no. of cells observed | 81 | 162 |
| No. of cells with laggards (%) | 16 (20) | 46 (28) |
| No. of cells with bridges (%) | 15 (19) | 48 (30) |

*Table 4.* Screening the hybrid plants for PBNV, PMV and PSTV by ELISA

| Disease | ELISA reading + control | ELISA reading – control | ELISA reading Hybrid plants |
|---|---|---|---|
| PBNV | 3.6 | 0.07 | 0.07 |
| PMV | 3.4 | 0.07 | 3.22 |
| PSTV | 3.34 | 0.07 | 0.08 |

+ control = ELISA reading when the plant is completely infected.
– control = ELISA reading of uninfected plant.

crude extracts taken from the leaves. ELISA test for the presence of PMV showed the presence of virus with an OD of 3.22 nearly as much as the positive control (3.4). The plants also showed mottling symptoms in younger leaves. The results of the ELISA tests have been tabulated in Table 4.

## Discussion

On the basis of their extensive collections and crossability studies in the genus *Arachis*, Gregory & Gregory (1979) grouped the accessions into seven sections. Krapovickas & Gregory (1994) grouped the wild species accessions into nine sections based on morphological characters, crossability and hybrid fertility. In both the classifications only sections *Arachis* and *Rhizomatosae* contain tetraploids. Most of the wild species within section *Arachis* are crossable with each other and with *A. hypogaea*. Incorporation of disease resistance genes from diploid wild species of the section *Arachis* has been the basis for genetic improvement of *A. hypogaea* (Singh, 1986; Stalker & Moss, 1987).

All the species of the section *Arachis* are diploid except *A. hypogaea* and *A. monticola*. Gene transfers from diploid species into tetraploid cultivated species involve several steps such as conversion of sterile triploids into fertile hexaploids followed by several backcross cycles to achieve tetraploid status.

Tetraploid wild species from section *Rhizomatosae* offer opportunities for single step production of tetraploid $F_1$ hybrids. Further, the accessions of section *Rhizomatosae* have resistances to a wider range of pests and pathogens (Moss, 1980; Moss et al., 1988) than those of section *Arachis*. These prompted studies on the barriers to inter-sectional crossability and on developing methods for the production of hybrids from such crosses. The barriers to hybridization between these species operate during fertilization and during post-fertilization development of the peg. It is possible to overcome these barriers by application of hormones to the pistils of intersectionally pollinated flowers (Sastri & Moss, 1982; Mallikarjuna & Sastri, 1985a) followed by rescue of hybrid embryos by *in vitro* culture (Mallikarjuna & Sastri, 1985b).

Although morphologically the hybrid between *A. hypogaea* and *A. glabrata* showed a mixture of distinct traits of both the parents, the acquisition of the disease resistance genes and the perennial growth habit were undoubtedly from the male parent. An interesting deviation from both parents was the morphology of the flower in their hybrid. Many flowers lacked wing petals, a few lacked standard or keel petals. Abnormal floral structure has been consistently observed. There is evidence that hybridization can sometimes induce transposable element activity (Schwarz-Sommer et al., 1985). Transposable element activity has been shown to be responsible for the appearance of novel floral patterns in some snapdragon crosses (Martin et al., 1988), which suggest molecular genetic interference in the functioning of genes for flower morphogenesis. During this process certain new DNA sequences are formed which do not remain fixed stably in the genome and move from one location to another, thus disrupting the normal functioning of certain genes such as those for petal formation as in the present case.

A perfect metaphase with 20 ring bivalents was conspicuously absent except in one cell. Maximum number of ring bivalents per pollen mother cell was 9.8 and a minimum of 6 was always present which was due to total homology between one of the genome of *A. hypogaea* and *A. glabrata*. A majority of the cells showed 4–6 univalents and 14–17 bivalents with a mean of 15.32 bivalents was due to meiotic associations in the hybrid between two putative donors of A and B genomes species (A and B genomes of *A. hypogaea*) and the R genome (RR of *A. glabrata*) which indicated the high degree of homeology between the genomes of the species involved in the cross.

Singh & Moss (1982) based on cytological studies, suggested *A. batizocoi* as the B genome species and *A. duranensis* as the A genome contributor to *A. hypogaea*. Meiotic observations of the hybrid revealed a mean of 15.3 bivalents and 9.5 univalents. A mean chiasma frequency of 5.52 rod bivalents indicate homeology of the three genomes involved – A, B and R (A and B genomes of *A. hypogaea* and R genome of *A. glabrata*).

Screening the hybrid plant for PBNV and PSTV revealed that disease resistance from *A. glabrata* was successfully transferred. Therefore, recombination and exchange of genetic material provide a means to determine that the genomes involved were not very divergent, and demonstrate that it is possible to access desirable traits from *A. glabrata.*

## References


Gregory, M.P. & W.C. Gregory, 1979. Exotic germplasm of *Arachis* L. interspecific hybrids. J Hered 70: 185–193.

Hobbs, H.A., D.V.R. Reddy, R. Rajeshwari & A.S. Reddy, 1987. Use of direct antigen coating and protein A coating ELISA procedures for detection of peanut viruses. Plant Diseases 71: 747–750.

Krapovickas, A. & W.C. Gregory, 1994. Taxonomia del genero *Arachis* (Leguminosae). Bonplandia 8: 1–186.

Mallikarjuna, Nalini & D.C. Sastri, 1985a. Utilization of incompatible species in *Arachis*: Sequential hormone applications. In: J.P. Moss (Ed.), Cytogenetics of *Arachis*, pp. 144–152. Proc. International Workshop on Cytogenetics of *Arachis*. ICRISAT, Patancheru, Andhra Pradesh, India.

Mallikarjuna, Nalini & D.C. Sastri, 1985b. *In vitro* culture of ovules and embryos from some incompatible interspecific crosses in the genus *Arachis* L. In: J.P. Moss (Ed.), Cytogenetics of *Arachis*, pp. 153–158. Proc. International Workshop on Cytogenetics of Arachis. ICRISAT, Patancheru P.O. 502 324, Andhra Pradesh, India.

Martin, C., S. Mackay & R. Carpenter, 1988. Large-scale chromosomal restructuring is induced by the transposable element Tam 3 at nivea locus of *Antirrhinum majus*. Genetics 119: 171–184.

Moss, J.P., 1980. Wild species in the improvement of groundnuts. In: R.J. Summerfield & A.M. Buntings (Eds.), Advances in Legume Science, pp. 525–535. Royal Botanic Gardens, Kew.

Moss, J.P., A.K. Singh, D.C. Sastri & I.S. Dundas, 1988. Wide hybridization in legumes at ICRISAT. In: J.M.J. de Wet (Ed.), Biotechnology in Tropical Crop Improvement, pp. 87–96. Proceedings of the International Biotechnology Workshop, 12–15 Jan., 1987, ICRISAT, Patancheru P.O. 502 324, Andhra Pradesh, India.

Sastri, D.C. & J.P. Moss, 1982. Effects of growth regulators on incompatible crosses in the genus *Arachis* L. J Exp Bot 33: 1293–1301.

Scandalios, J.G., 1969. Genetic control of multiple molecular forms of enzymes in plants. A review. Biochem Genet 3: 37–76.

Schwarz-Sommer, Z., G. Cutpers, P.A. Peterson & H. Saedler, 1985. Plant transposable elements generate the DNA sequence diversity needed in evolution. EMBO J 4: 591–599.

Singh, A.K., 1986. Utilization of wild relatives in the genetic improvement of *Arachis hypogaea* L. Theor Appl Genet 72: 433–439.

Stalker, H.T. & J.P. Moss, 1987. Speciation, cytogenetics and utilization of *Arachis* species. Advances in Agronomy 41: 1–40.

Subrahmanyam, P., D. McDonald, R.W. Gibbons, S.N. Nigam & D.J. Nevill, 1982. Resistance to rust and late leafspot diseases in some genotypes of *Arachis hypogaea*. Peanut Science 9: 6–10.