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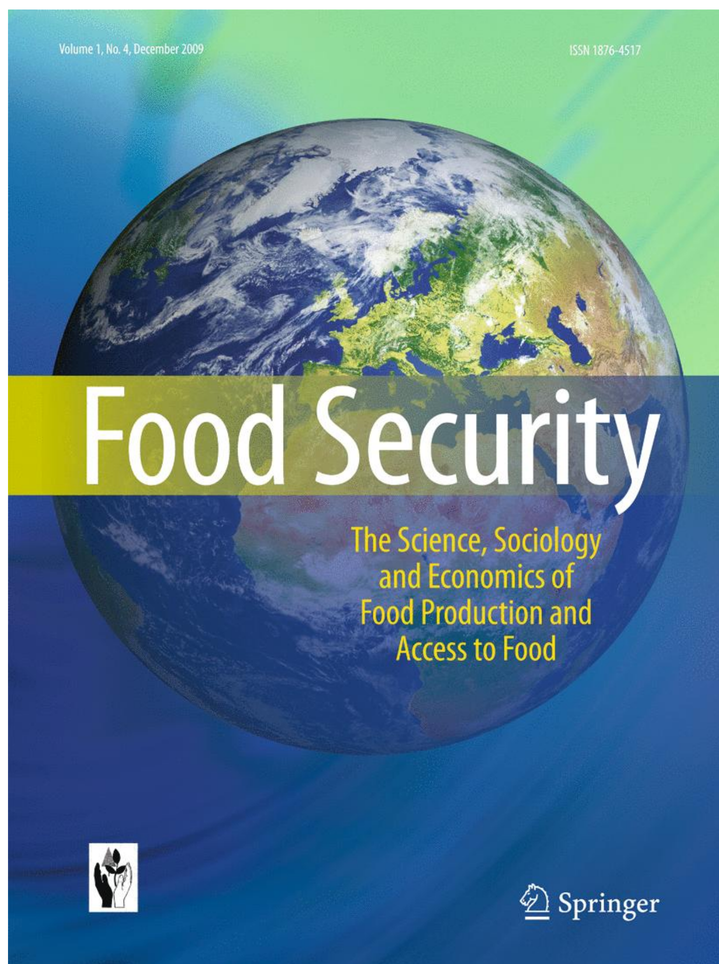

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ORIGINAL PAPER

Peanut improvement: production of fertile hybrids and backcross progeny between *Arachis hypogaea* and *A. kretschmeri*

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Abstract There are only a few reports of successful crosses between cultivated peanut (*Arachis hypogaea* L., section *Arachis*) and wild species from sections other than section *Arachis*. Many of the wild *Arachis* species harbor important traits necessary for the improvement of peanut. For example, *Arachis kretschmeri* Krapov., W.C. Gregory & C.E. Simpson (section *Procumbentes*) can grow under water-logged conditions and has been identified as one of the few wild species of *Arachis* with resistance to late leaf spot (LLS) and peanut rosette disease. Peanut rosette, caused by a combination of viruses, is an economically important disease only in Africa, while LLS, caused by *Cercosporidium personatum*, is an important fungal disease in Asia and the Americas as well as Africa. Interspecific hybrids between *A. hypogaea* and *A. kretschmeri* were produced by applying growth regulators to pollinated pistils and hybrid plants were obtained by germinating embryos in vitro. A total of seven hybrids were produced and confirmed by Simple Sequence Repeat (SSR) analysis. All hybrids were fertile, although initially slow growing. F_1 hybrids were backcrossed to *A. hypogaea* and all plants in the F_1BC_1 generation were single-seeded with a prominent beak, characteristic of *A. kretschmeri*, but many of the F_1BC_2 pods were double-seeded resembling *A. hypogaea*. F_1BC_2 plants were moderately resistant to LLS. When a large number of seeds are obtained, the progeny will be screened for resistance to both LLS and rosette disease. Thus crosses with species outside the section *Arachis* may not only confer disease resistance but will also broaden the genetic base of cultivated peanut.

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Keywords Groundnut · Wild species · *Arachis kretschmeri* · Interspecific hybridization · Embryo germination

Introduction

Asia and Africa account for a major portion of the world's groundnut (*Arachis hypogaea* L.) production. Groundnut is grown by small holder farmers under rain fed conditions with limited inputs. Production is lower than expected in both Asia and Africa due to a number of diseases that reduce yield. Methods to control diseases are beyond the reach of resource poor farmers. Two of the most important diseases that reduce yields are rosette in Africa and late leaf spot (LLS) in Asia, Africa and the Americas.

Groundnut is the principal source of human dietary protein, oil/fat and vitamins such as thiamine, riboflavin and niacin in parts of Asia and Africa (Savage and Keenan 1994). Groundnut paste is an important source of calories for small children, particularly those being weaned. These children cannot obtain the calories they require from high-bulk cereal grains and depend on groundnut for energy as well as vitamins. Groundnut cake and haulms (straw, stems) are used as livestock feed and help to maintain livestock productivity. The crop also contributes up to 60 kg/ha nitrogen to the soil, benefiting crops subsequently planted in the same field (Sprent 1994).

Groundnut rosette disease is found in Africa but rarely occurs in other parts of the world. It is the most destructive

virus disease of groundnut in sub-Saharan Africa, causing losses of around 156 million US$ (Dwivedi et al. 2003). The disease is aphid transmitted and is caused by a complex of three agents; Groundnut rosette assistor virus (GRAV), Groundnut rosette virus (GRV) and its satellite RNA (sat. RNA). Management of groundnut rosette disease by insecticidal control of the vector has been recognized since the mid-1960s. Cultural practices such as early sowing at optimal plant densities reduce the incidence of the disease but smallholder farmers in Africa, for a number of reasons, seldom adopt these practices. Therefore, host-plant resistance to the disease is regarded as the most viable and sustainable solution.

Late leaf spot (LLS), caused *by Cercosporidium personatum*, is an important foliar disease of groundnut in Africa, Asia and the Americas. An estimated global loss in yield of 600 million US$ due to LLS has been reported (Dwivedi et al. 2003). Hence, yield losses due to the disease can be a major impediment to groundnut production. Managing the disease through the application of fungicides is not a viable option for resource poor farmers. Besides, the application of fungicides may pollute the environment, including ground water, thus causing greater risk and damage than the loss of the crop due to the disease.

Molecular analysis has shown that the crop has a narrow genetic base (Halward et al. 1991; Hopkins et al. 1999). A principal reason for this may be that a single hybridization event gave rise to the tetraploid cultivated peanut some 3,500 years ago (Kochert et al. 1996). There is, however, much molecular variation in the nine different sections of *Arachis* (Mallikarjuna et al. 2005; Milla et al. 2005). Wild species from the section *Arachis* have been used in the improvement of cultivated species (Stalker et al. 1991; Singh 1985; Mallikarjuna et al. 2004a, b). Simpson and Starr (2001) released the first root-knot nematode resistant peanut cultivar COAN using *A. cardenasii* Krapov. and W. C. Gregory. Wild species in the other eight sections are incompatible with the cultivated peanut and specialized techniques are required for crossing (Mallikarjuna and Sastri 1985, 2002; Mallikarjuna 2003).

Arachis kretschmeri Krapov., W.C. Gregory & C.E. Simpson, a member of section *Procumbentes*, is a native of Mato Grosso do Sul, Brazil. *A. kretschmeri* can grow in waterlogged conditions (Krapovickas and Gregory 1994) and is resistant to late leaf spot and groundnut rosette disease (Subrahmanyam et al. 2001). Utilization of *A. kretschmeri* in an *A. hypogaea*-improvement program could contribute resistance to LLS and rosette disease in cultivated varieties and would broaden the genetic base of the crop. Groundnut resistant to rosette and LLS would not only increase production but would contribute to food security and income generation for the resource poor farmers of Asia and Africa.

Materials and methods

Seeds of *A. kretschmeri* with $2n=2x=20$, (ICG 8191; collector number 30007; PI 468151) were obtained from the genetic resources division of ICRISAT and grown in a glasshouse (Fig. 1a). Seeds of *A. hypogaea* cv. ICGV 87128 ($2n=4x=40$) (Nigam et al. 1990) were also grown and maintained in the glasshouse. Flowers were emasculated a day before pollination and cross pollination, using *A. hypogaea* as the female parent and *A. kretschmeri* as the pollen donor, was carried out before 10:00 am on the following day. Application of gibberellic acid (GA_3) (0.5 ml; 75 mg/L) by means of a cotton swab impregnated with the hormone and wrapped around the base of pollinated pistils was mandatory for obtaining pods from crosses.

Immature pods from cross-pollination were harvested 18–20 days after pollination, surface-sterilized and ovules were extracted under sterile conditions. Ovules that were between 4.0 and 5.0 mm long were dissected and embryos (immature seeds) were cultured directly on semi-solid growth medium consisting of MS (Murashige and Skoog 1962) basal medium with 3% sucrose plus naphthalene acetic acid (NAA; 0.1 mg/L) and benzylamino purine (BAP; 1.0 mg/L). Cultures were incubated at 24°C with a photoperiod of 10 hr light and 14 hr darkness and under 45–50% relative humidity.

Embryos germinated and gave rise to seedlings with individual or multiple shoots. Shoots were rooted in vitro on rooting medium consisting of half strength MS basal salts plus 3% sucrose, NAA (2.0 mg/L) and indole-3-butyric acid (IBA; 1.0 mg/L). After 15 d on rooting medium, shoots were transferred to half strength MS basal medium without growth regulators. Healthy roots developed within 3 weeks of transfer. Shoots with well-developed roots were transferred to sand, watered with sterilized tap water and acclimatized under controlled conditions at 24°C and relative humidity of 72–75%. A month of acclimatization was sufficient before transfer to the glasshouse. In the glasshouse, plants were transferred to pots filled with a steam sterilized potting mixture consisting of soil, sand, and farm yard manure (4:1: 1).

DNA was extracted from young leaflets using Qiagen miniprep kits (Qiagen, Valencia, CA) according to the manufacturer's protocols. For simple sequence repeat (SSR) analysis, the procedure and primers described in detail in Ferguson et al. (2004) were followed. PCR reactions contained 30 pmol primers, 5 ng template DNA, 4 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* polymerase with 1 X 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

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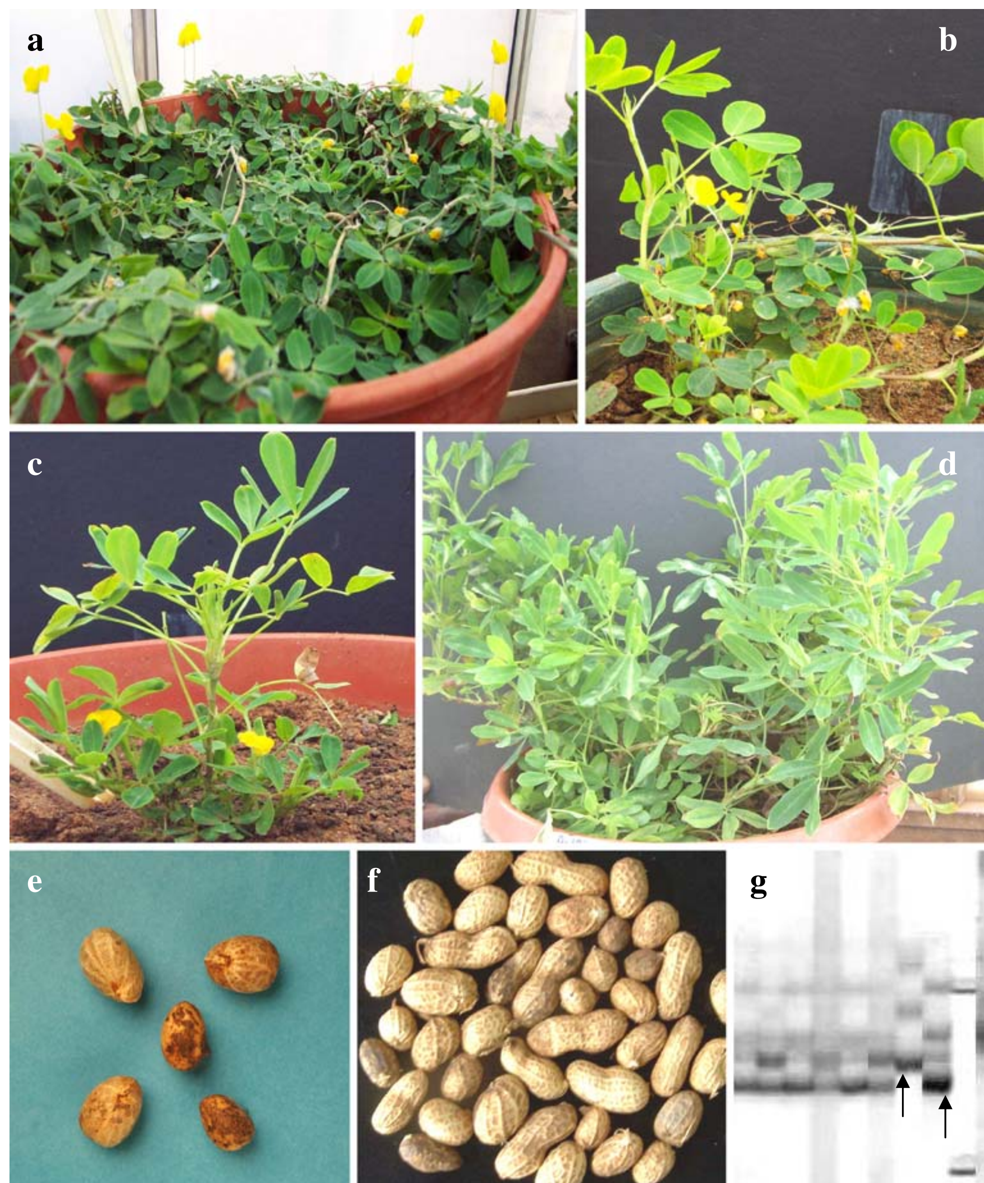

Fig. 1 Interspecific hybridization of *Arachis hypogaea* x *A. kretschmeri* (section *Procumbentes*). **a** Wild species of *Arachis kretschmeri* used as the pollen donor. **b** F_1 hybrid between *A. hypogaea* cv ICGS 44 × *A. kretschmeri*. **c** Backcross (BC_1) from the cross *A. hypogaea* × *A. kretschmeri*. **d** BC_2 hybrid from the cross *A. hypogaea* × *A. kretschmeri*. **e** Pods from BC_1 hybrid from the cross *A. hypogaea* × *A. kretschmeri*. **f** Pods from BC_2 hybrid from the cross *A. hypogaea* × *A. kretschmeri*. **g** SSR primer 2A06 analysis of F_1 hybrids from the cross *A. hypogaea* × *A. kretschmeri.* The *arrow* on the second lane from the *left* points to the unique band present in *A. kretschmeri* and the *arrow* on the third lane points to the unique band of *A. hypogaea*

of 10 min at 72°C. Amplification products were visualized on non-denaturing 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 followed by development in a 1.5% NaCl solution with 0.02% by volume formaldehyde solution.

The ploidy of the derivatives was determined by pollen diameter analysis (Singsit and Ozias-Akins 1992). Three classes were observed: that of the diploid species, *A. kretschmeri*, was 25 to 27 μm; that of *A. hypogaea* (tetraploid species) was 45 to 47 μm and that of triploid interspecific derivatives was 27 to 29 μm. The pollen diameters of triploids which had undergone 2*n* restitution (2n=40), was 43 to 45 μm, comparable with pollen grains of tetraploid plants.

Pollen grains were isolated from flowers on the day of anthesis. Pollen fertility counts were made on pollen grains stained with 2% acetocarmine. Grains stained dark pink were counted as fertile and unstained grains with irregular shape were counted as sterile. The data were collected from counts taken on three different days during the flowering period.

Screening for LLS resistance was carried out in the field under unprotected field conditions. After 21 days of growth, leaves infected with spores were stapelled to the test plants and disease reaction noted after 95 days. LLS screening was also carried out using the detached leaf technique. Cultivar TMV 2 was used as the susceptible

check. Plastic trays with autoclaved sand were used to place tetrafoliate leaves in a randomized block design with 3 replications. LLS spores were harvested with a cyclone spore collector. The concentration of the suspension was 20,000 spores/ml. A few drops of Tween 80 (polyoxyethylene sorbitan mono-oleate) were added. Spore suspension was used to spray inoculate the leaves. Immediately after inoculation, leaves were placed in a growth room at 23–25°C to ensure wetness of the leaf surface during the night. Leaves were observed for sporulation, percent leaf damage due to sporulating colonies and time taken to sporulation. Based on these parameters damage due to LLS was calculated at the end of 30 days.

Results and discussion

Crosses between *Arachis hypogaea* cv ICGV 87128 and *A. kretschmeri* yielded 51% pod set from a total of 184 pollinations. All pods set as a result of cross-pollination were immature and could be classified into two categories. In one category (63% of the hybrid pods), seeds inside the pods were less than 3 mm in length. These pods were not used in the study, as they would require in-ovulo embryo rescue techniques (Mallikarjuna 2003), which are time consuming processes. In the second category (37% of the pods), seeds were large although they were immature with thin papery cotyledons. Hybrid plants were obtained if the decoated seeds were germinated directly in vitro soon after harvesting and without drying the seeds, but the seeds did not germinate following normal harvesting and drying, even if in vitro germination was used. In the present investigation embryos/decoated seeds which were approximately 4–5 mm in length, were directly cultured/germinated on Murashige and Skoog's (MS) medium with 3% sucrose, 0.7% agar, NAA 0.1 mg/L and BAP 1.0 mg/L (Mallikarjuna 2005).

Seven hybrid plants were obtained as a result of in vitro germination. The hybrids had intermediate morphology with a semi-spreading growth habit (Fig. 1b). Morphology of the leaves was intermediate between the two parents. Two SSR markers (2A06 and 4H11) were polymorphic between the two parents. All seven F_1 hybrids used in the study had both male and female parent specific bands (Fig. 1g). The hybrid plants had slow growth to begin with, although later they grew and flowered profusely. Pollen fertility in the F_1 hybrids was low (8–16%). F_1 hybrid plants were used as the female parent and crossed with cv. ICGV 87128 as the male parent. Pegs were observed on all hybrid plants but pods were obtained on only two plants. A total of 13 pods were obtained in a span of 4 months as a result of 297 pollinations. All the pods were single seeded. Two seeds germinated giving rise to BC_1F_1 plants (Fig. 1c). Pollen fertility in the BC_1F_1 plants ranged from 18–32%. Pods from BC_1F_1 plants were also single seeded (Fig. 1e). The second backcross (BC_2F_1) plants were obtained using the hybrid as the female parent (Fig. 1d), produced many double seeded pods that resembled *A. hypogaea* pods (Fig. 1f). Pollen fertility in the BC_2F_1 plants was 45–70%. BC_2F_1 plants were screened for LLS resistance under field conditions. All plants showed 20–30% defoliation with 10% leaf area damage. At 96 days after inoculation, all the leaves defoliated on the cultivated parent control (cv ICGV 87128) due to heavy sporulation. The test plants had 5–6 spots on the leaves. Hence they were classified as moderately resistant to LLS. In the detached leaf experiment, the test plants had a longer incubation period of 14–15 days compared to 7 days in the control cultivar (TMV 2). Although the lesions were large, the lesion number on the 30th day after inoculation varied from 4 to 6 compared to 50–55 on the susceptible control (Fig. 2). Also, the area of leaf damage at 30 days after inoculation was 10 to 12% compared to 60 to 66% on the susceptible control. Infection frequency (number of lesions per square centimeter of leaf area) was calculated at 0.25 compared to 1.8 on the susceptible control, demonstrating moderate level of resistance to LLS. Once large numbers of seeds have been generated, the material will be screened for LLS resistance under field conditions at Patancheru, India and for groundnut rosette resistance at a hot spot location on the African continent as rosette does not occur in Asia.

Many of the wild species from section *Arachis* have been successfully crossed with *A. hypogaea* and hybrids obtained (Stalker et al. 1991; Singh 1985; Mallikarjuna et al. 2004a, b) and various introgression schemes have been used to obtain backcross progeny (Simpson 2001). In the present experiment, the triploid F_1 hybrid was used as the female parent and crossed with *A. hypogaea*. Although a

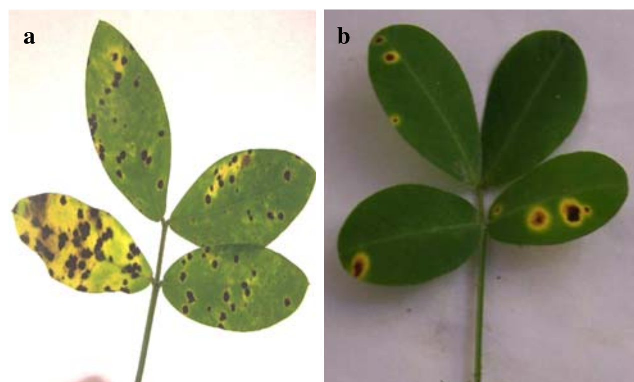

Fig. 2 Late leaf spot reaction in the progeny between *A. hypogaea* x. *A. kretschmeri*. **a** Susceptible LLS reaction in cv TMV2. **b** Progeny from the cross *A. hypogaea* x *A. kretschmeri* showing moderate level of resistance to LLS

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large number of pollinations were necessary to obtain pods, the approach resulted in the production of tetraploid BC_1F_1 (Fig. 1c) hybrid plants within a short time scale.

Both *n* and 2*n* pollen were observed in the F_1 hybrid plants, demonstrating that there had been restitution of chromosomes at meiosis. Because the egg is embedded deep in the ovular tissue, analysis of pollen grains at the tetrad stage was examined and gave a good indication of the formation of 2n gametes. Many dyads were observed, which resulted from 2*n* pollen. The observation of tetraploid BC_1F_1 plants is a good indication that 2n gametes are also formed in the egg.

Arachis kretschmeri, when crossed with *A. hypogaea*, produced fertile plants. It was possible to successfully backcross the hybrid plants and obtain F_1BC_1 and F_1BC_2 plants. F_1BC_2 hybrids set greater numbers of pods than either F_1 or F_1BC_1 hybrids and the pods resembled those of *A. hypogaea.*

It was possible to obtain hybrids between *A. hypogaea* and *A. chiquitana*, another wild species from section *Procumbentes* (Mallikarjuna 2005). These results show that some wild species in section *Procumbentes* can be successfully crossed with *A. hypogaea* using in vitro techniques.

Arachis glabrata from section *Rhizomatosae* has been successfully crossed with *A. hypogaea* using in vitro techniques (Mallikarjuna and Sastri 2002) and traits of interest such as resistance to late leaf spot and groundnut viral diseases caused by peanut mottle virus (PMV), peanut stripe virus (PSTV) and peanut bud necrosis virus (PBNV) transferred (Mallikarjuna 2003). In the present study, it was possible to transfer moderate level of LLS resistance from *A. kretschmeri* and the potential to obtain rosette resistance in the progeny will be known once the material has been screened for rosette in Africa. The significance of crossing wild species from other sections is that increased numbers of *Arachis* species become available for the introduction of useful characters into cultivated groundnut. Also, the relationship between different sections will become clearer as the classification of the genus is based on the crossability (Krapovickas and Gregory 1994). More importantly, such materials broaden the genetic base of the crop.

Late leaf spot resistant groundnut would have an impact on the groundnut growing regions of Andhra Pradesh and also in other parts of India and later in other groundnut growing regions of the world. It needs to be seen if the progeny have other desirable traits as crossing with wild relatives reorganizes the whole genome apart from adding exotic genetic material (Hoisington et al. 1999).

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Nalini Mallikarjuna Ph.D I am interested in gene introgression from wild relatives, from compatible and incompatible gene pool, to broaden the genetic base and introduce useful traits from wild relatives of groundnut, chickpea and pigeonpea. I am presently involved in the development of synthetic groundnuts or new sources of *Arachis hypogaea* and using them to broaden the genetic base of groundnut and introduce fungal disease resistance. The emphasis in pigeonpea has been to cross wild relatives to introgress resistance to pod borers, resistance to some important pigeonpea diseases and identify new sources of cytoplasmic male sterility. Closely related wild species are being used in chickpea to introgress divergent genes for different traits and to broaden the genetic base of the crop.

Dr. Dave Hoisington Currently, the Deputy Director General for Research at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) with oversight of ICRISAT's global research and development activities in sorghum, millets, chickpea, groundnut and pigeonpea cropping systems. Have published over 70 refereed journal articles, 30 book chapters and made numerous invited presentations at international conferences. Is a member of the Editorial Board for *Theoretical and Applied Genomics* and *International Journal of Plant Genomics*. Joined ICRISAT in March 2005 as the Global Theme Leader for Biotechnology. Prior to ICRISAT, served in various positions at the International Maize and Wheat Improvement Center (CIMMYT) leading the Genetic Resources Program and the Applied Biotechnology Center for nearly 16 years. Prior to working at CIMMYT, was an Assistant Research Professor in the Agronomy Department at the University of Missouri. Obtained a BS in Botany and Plant Pathology from Colorado State University, Ft. Collins and a PhD in Plant Biology from Washington University, St. Louis.