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Use of Hormones and Ovule and Embryo Culture to Enhance Wide Crosses in *Arachis*

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Introduction

A plant species maintains its identity by allowing gene exchange between members of the species, but preventing gene exchange with members of other species. Similarities between closely related species decrease with more distantly related species or genera, and at the same time there is usually a decrease in the ease of producing hybrids and the meiotic regularity and fertility of any hybrids that can be produced. Although breeders have relied almost exclusively on intraspecific gene transfer, and on meiotic recombination in the F_1 hybrids, to produce the vast majority of cultivated plants, many of these wild species are resistant to pests and diseases, and form a valuable gene pool.

The wild relatives of crop plants contain a wealth of desirable characters, not only resistance to stresses such as diseases, pests or inclement conditions, but also desirable growth attributes (Frey, 1983). Some wild *Arachis* species are resistant to important yield-reducing pests and diseases, and a few species contain multiple resistance (Stalker and Moss, 1987). Often these species are isolated from crop plants by mechanisms that are effective in nature, but can be overcome by various techniques. The resulting F_1 hybrids may be crossable with the cultivated species, or further manipulations may be necessary to produce segregating F_2 populations, but the resulting derivatives can be very valuable, either as potential cultivars, or as new germplasm (Moss, 1985).

The natural barriers to interspecific hybridization that have evolved to maintain the identity of a species can be classified into prefertilization and postfertilization. The barriers may be single and simple, controlled by a simple gene system for which variation exists in the form of crossable species that can be easily exploited, or may be complex, as there are many processes involved in sexual reproduction. These include pollen recognition, pollen germination, pollen tube penetration, pollen tube growth through the style, penetration of the micropyle, fertilization, endosperm development, embryo development, fruit development, and seed maturation. The chemistry and morphology of the flower, pollen, stigma, style, fruit, and seed differ between genera, and truly wide sexual hybrids may

always fail due to these differences. A range of techniques have been reported as effective in overcoming barriers to hybridization.

The *Arachis* flower is a modified legume flower, where the calyx, corolla, and anthers are borne at the top of an elongated tubular hypanthium, at the base of which is the ovary. The style extends the full length of the hypanthium tube, and the stigma is positioned between the anthers. After fertilization, a meristem is formed at the base of the ovary, and a gynophore or "peg" grows into the soil, and the pod is formed under the soil. Geotropic response and elongation of the peg is dependent on the presence of the meristem, and is not dependent on the presence of the ovary (Ziv and Zamsky, 1975).

Interspecific crosses are of interest to the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), as certain wild species contain resistances to important pests and diseases (Moss, 1985) for some of which resistance is not available in cultivated groundnut. A number of derivatives have been produced from interspecific crosses and used by breeders, but genes from some species have not yet been introgressed into the cultivated groundnut.

The genus *Arachis* has been classified into seven sections (Gregory and Gregory, 1979). Section *Arachis* comprises tetraploid *A. hypogaea*, the cultivated groundnut or peanut, and one tetraploid and a number of diploid wild species. Section Rhizomatosae comprises a diploid species, and one or more tetraploid species, which are rhizomatous perennials. These are resistant to many pests and diseases (Moss, 1988; ICRISAT, 1987), and production of hybrids has a high priority in the ICRISAT Cytogenetics Unit. Use of mentor pollen, and mentor pollen leachate, with or without hormones, showed that hormone application at the time of pollination was an effective means of stimulating hybrid peg formation (Sastri and Moss, 1982).

This paper describes techniques whereby hybrid embryos were routinely produced and stimulated to develop to the stage where they could be cultured, either to produce callus for regeneration of hybrid shoots, or to produce seedlings.

Materials and Methods

The species and accessions used in the main study are listed in Table 1. Other species are listed in Table 3. *A. hypogaea* was used as female parent, as *Rhizomatosae* species produce few seeds. Female plants were emasculated in the evening and pollinated the

following morning, the standard technique for groundnut (Norden, 1980). Hormones used to induce peg and pod production were kinetin (6-furfurylaminopurine) (Kn), indole-3-yl acetic acid (IAA), 1-naphthyl acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), and gibberellic acid (GA). Kn, NAA, IAA, 6-benzyl amino purine (BA) and indole butyric acid (IBA) were used in culture media.

Table 1. Identity and Source of Taxa Used in Crossing Program.

Female parents
Section *Arachis*
Arachis hypogaea
ssp. *hypogaea* var. *hypogaea*
cv M 13 (Punjab Agricultural University, Ludhiana, India)
cv MKI 374 (Kadiri, India)
cv Robut 33-1 (Kadiri, India)
ssp. *fastigiata* var. *vulgaris*
cv TMV 2 (Tirivandum, India)
Male parent
Section *Rhizomatosae*
Arachis sp. PI 276233[1] (Reading University, U.K.)[2]

[1]PI = USDA Plant Inventory Number.
[2]Original collection from Paraguay.

Hormones were applied at the time of pollination by immersing cotton wool in aqueous hormone solutions, and wrapping the wetted cotton wool around the base of the hypanthial tube. Hormones were applied to the peg by wrapping hormone-wetted cotton wool around the peg at the soil surface, or applying hormones in lanolin to the base of the peg.

Sixty days after pollination, pegs or immature pods were collected, surface sterilized, and ovules excised. Ovules were cultured on filter paper bridges over liquid MS medium (Murashige & Skoog, 1962) with 3% sucrose, 0.1 mg L^{-1} Kn and 0.075 mg L^{-1} IAA until embryos had grown out of, or could be excised from, the ovules. Heart-shaped or early cotyledonary embryos were cultured on MS medium with 3% sucrose, 0.01 mg L^{-1} NAA and 0.1 mg L^{-1} BA to stimulate further growth into seedlings, but the less-developed globular embryos were cultured on MS with 3% sucrose, 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA to simulate callus formation and subse-

quent shoot production. Shoots were rooted on filter paper supports in liquid MS with 3% sucrose, 2 mg L^{-1} NAA and 1 mg L^{-1} IBA, and rooted shoots transferred to soil in pots. Unrooted shoots were excised from callus and grafted onto parental stocks.

Results

Effects of Hormones on Peg Production

The effect of five hormones (Kn, IAA, NAA, 2,4-D, and GA) on peg production was tested (Table 2).

Table 2. Effect of Hormones on Peg and Pod Production When *Arachis hypogaea* is Pollinated with *A.* sp. 276233.

Treatment	No. of Pollinations	Pegs/ Pollination (%)	Pods/ Peg (%)
Control	85	15	0
Kn 0.02 ppm	57	44	68
Kn 0.2 ppm	89	23	50
Kn 2.0 ppm	28	36	90
IAA 10 ppm	88	9	25
25 ppm	79	19	0
50 ppm	81	5	0
NAA 10 ppm	52	19	80
25 ppm	95	33	45
50 ppm	27	4	0
2,4-D 25 ppm	54	11	0
GA 25 ppm	144	56	20

Control (water) resulted in 15% peg production. Kn and GA were consistently better at all concentrations tested, and GA at 44 ppm stimulated the most pegs (86%). Although NAA at 25 ppm gave 33% pegs, other concentrations and the other auxins tested, IAA and 2,4-D, did not increase peg production appreciably, or produced fewer pegs than the controls.

Effect of GA on Other Interspecific Crosses in Arachis

GA treatment was used in a range of interspecific and intersectional crosses in *Arachis*, which do not usually produce pegs or pods (Table 3). Section *Triseminale* (2n = 20) has remained isolated from the rest of the genus (Gregory & Gregory) but produced 79% pegs, 39% of which formed pods, when crossed with *A. duranensis*

Table 3. Peg and Pod Production after Gibberellin Treatment in Some Intersectional Crosses.

	Pollinations (no.)	Pegs/Poll (%)	Pods/Peg (%)
Section *Arachis* x Section *Triseminale*			
A duranensis (2n = 20) x *A pusilla* (2n = 20)	33	79	39
A hypogaea cv Robut 33 1 x *A pusilla* (2n = 20)	78	46	0
Section *Arachis* x Section *Erectoides*			
A hypogaea cv Robut 33 1 x *A rigonii* (2n = 20)	45	64	13
A hypogaea cv M 13 x *A rigonii*	18	94	22
A hypogaea cv TMV2 x *A rigonii*	43	86	26
Section *Arachis* x Section *Extranervosae*			
A hypogaea cv Robut 33-1 x *A villosulicarpa* (2n = 20)	39	59	3
A hypogaea cv MK 374 x *A villosulicarpa* (2n = 20)	9	89	11
Section *Extranervosae* x Section *Triseminale*			
A villosulicarpa (2n = 20) x *A pusilla* (2n = 20)	24	54	46
Section *Arachis* x Section *Rhizomatosae*			
A hypogaea cv Robut 33-1 x *Arachis* sp Coll 9649	82	44	6
A hypogaea cv Robut 33 1 x *Arachis* sp Coll 9797	46	57	2
A hypogaea cv Robut 33-1 x *Arachis* sp Coll 9806	26	62	0
A hypogaea cv Robut 33-1 x *Arachis* sp Coll 9813	7	30	50
A hypogaea cv TMV2 x *Arachis* sp Coll 9649	11	73	0
A hypogaea cv MK 374 x *Arachis* sp Coll 9649	26	42	15
A hypogaea cv MK 374 x *Arachis* sp Coll 9797	14	65	0
A hypogaea cv M 13 x *Arachis* sp PI 276233	75	56	5
A hypogaea cv Chico x *Arachis* sp PI 276233	58	66	9
A hypogaea cv Chico x *Arachis* sp PI 9649	26	73	19

(2n = 20). Peg production was lower in the cross with *A. hypogaea*, and no pods were formed. *A. hypogaea* produced pegs and pods when pollinated with species from sections *Erectoides and Extranervosae*.

Pod Production

Pegs that have been produced following GA treatment at the time of pollination either failed to reach the soil, or failed to produce pods.

Four cultivars, MK 374, Robut 33-1, M 13, and TMV 2 were pollinated by *A.* sp. 276233. GA was applied at pollination, and 10, 25, 50 or 100 ppm IAA was applied in lanolin to the developing peg 10, 15, 20, or 25 days after pollination (Table 4). The percentage pod formation without IAA treatment ranged from 10 (M 13) to 37 (TMV 2). After IAA treatment, percentage pod formation ranged from 0 (M 13) to 83 (TMV 2). IAA increased the number of pods formed in all cultivars. IAA had little overall effect in Robut 33-1, but in the other cultivars, mean pod production at any one time of application or at any concentration was higher than the controls. There was no significant difference between the concentrations used, though the highest pod production was usually after treatment with 50 or 100 ppm IAA. The difference between times of application was more marked, in most cases 20 or 25 days after pollination being the best time.

The effect of other phytohormones on production of pods on GA-induced pegs was also tested. Kinetin at 0.02 ppm applied to Robut 33-1 10 days after pollination increased pod production, but other times of application were not as good as with IAA. Similar results were obtained using MK 374. Kinetin applied to TMV 2 did not increase pod formation.

NAA was applied to pegs induced by GA on Robut 33-1 pollinated with A. sp. 276233 (Table 5). Results were better than when Robut 33-1 was treated with IAA.

Sequential Hormone Treatments to GA-induced Pegs

Two or three applications of IAA or Kn were compared with single treatments (Table 6). IAA applied 10 and 17 days after pollination was better than a single treatment at 10 days, but all the other multiple treatments decreased the number of pods produced.

Table 4. Frequency of Pod Formation on GA-induced Pegs of *A. hypogaea* x *A.* sp. 276233 after Treatment with Different Concentrations of IAA in Lanolin.

A. hypogaea Cultivar	IAA Conc (ppm)	Pods/Peg (%)				
		Time of Application (Days After Pollination)				
		10	15	20	25	Mean
MK 374	Control[1] = 20					
	10	42	43	67	48	48
	25	53	39	69	32	44
	50	25	32	56	34	36
	100	49	37	38	51	43
	Mean	43	38	56	41	
Robut 33-1	Control = 23					
	10	10	14	18	9	13
	25	27	9	20	14	17
	50	14	13	16	27	16
	100	31	18	8	40	20
	Mean	20	13	17	15	
M13	Control = 10					
	10	13	31	16	31	23
	25	8	33	27	6	20
	50	NA[2]	27	35	22	29
	100	(0)[3]	7	43	50	36
	Mean	11	26	32	25	
TMV 2	Control = 37					
	10	40	55	48	76	52
	25	39	53	65	47	50
	50	21	83	56	81	61
	100	85	31	69	69	57
	Mean	43	51	59	68	
Grand Mean		32	30	37	35	

[1]Control = GA treatment at pollination.
[2]NA = Not Attempted.
[3]( ) = less than 10 pollinations

Ovule and Embryo Sizes

Very few of the pods induced by IAA or NAA on GA-induced pegs after cross pollination matured, and such pods did not contain viable seeds. Immature pods were harvested, but embryos were too small to dissect and culture. However, ovules could be excised and cultured.

Table 5. Frequency of Pod Formation on GA-Induced Pegs on Robut 33-1 Pollinated with *A.* sp. 276233 after Treatment with NAA in Lanolin.

NAA Conc (ppm)	Pods/Peg (%)			
	Time of Application (Days after Pollination)			
	10	15	20	25
Control = 24				
10	48	44	37	42
25	61	41	35	35
50	12	33	22	77
100	39	52	40	30

Table 6. Effect on Sequential Applications of Hormones on Pod Production on GA-Induced Pegs in Robut 33-1 x *A.* sp. 276233.

Hormone	Pods/Peg (%)						
	Time of Application (Days after Pollination)						
1st Application	10	15	20	10	10	15	15
2nd Application				17	17	22	22
3rd Application					24		29
Control[1] = 19							
IAA (25 ppm)	27	11	16	32	3	6	8
Kn (0.02 ppm)	50	19	24	8	15	0	5

[1]Control = GA treatment at pollination.

A. hypogaea was crossed with *A.* sp. 276233, treated with GA, and developing pegs treated with different concentrations of IAA 10, 15, 20 or 25 days after pollination. The number of pods produced was consistent with other results; and ovules and embryos were excised 60 days after pollination and measured (Table 7). Ovule size was increased by some of the treatments, the best being 25 ppm 20 days after pollination. Concentrations 25 ppm or higher applied within 20 days after pollination were most effective. Embryo size was increased by all treatments. Although these embryos could be dissected from the ovules and cultured, culture of whole ovules gave better overall success rates.

Ovule Culture

Ovules were dissected from developing pods 60 days after pollination and cultured on filter paper bridges over liquid MS medium

Table 7. Effect of IAA in Lanolin on Pod-set and Ovule and Embryo Development in Gibberellin-induced Pegs in *A. hypogaea* cv. Robut 33-1 x *Arachis* sp. PI 276233.

Concentration of IAA (ppm)	Time of Application (Days after Pollination)			
	10	15	20	25
	Pod/Peg (%)[1], Control = 16%			
10	21	32	29	16
25	14	14	27	19
50	15	21	30	33
	Ovule length (mm), Control = 2.3 mm			
10	2.9	2.1	3.1	1.9
25	3.2	2.3	3.5	2.5
50	2.5	3.3	2.5	2.6
	Embryo length (mm), Control = 0.27 mm			
10	1.5	0.6	0.6	
25	2.6	1.0	1.5	0.6
50	1.0	0.9	0.9	0.6

[1]Total pegs treated = 368; minimum number in any treatment = 22.

supplemented with Kn and IAA (Table 8). Lower concentrations of IAA were more effective, though there was little difference in the final numbers of embryos that grew, as from 6 to 9 percent of ovules produced embryos that grew when excised and cultured.

Table 8. Response of *A. hypogaea* TMV2 x *A.* sp. 276233 Hybrid Ovules and Embryos When Cultured on Filter Paper Bridges over MS Medium with 0.1 ppm Kn and Different Concentrations of IAA.

IAA Conc (ppm)	Number of				% Ovules Producing Viable Embryos
	Ovules		Embryos		
	Cultured	Growing	Cultured	Growing	
0.0	138	110(80)[1]	38	13(34)	9
0.1	54	29(54)	11	4(36)	7
0.2	275	149(54)	47	16(34)	6
0.5	72	31(43)	24	6(25)	8

[1]Figures in parentheses are percentages.

Embryo Culture

Ovules were cultured on filter paper bridges over MS medium with 3% sucrose, 0.1 ppm Kn, and 0.075 ppm IAA. After one month of ovule culture, embryos were dissected from the ovules and cultured on a range of media to induce callus formation or to stimulate normal embryo growth and germination. However, the latter was only possible with heart-shaped and early cotyledonary embryos. On the

media used, MS + 3 or 5% sucrose, with either 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} or 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA, all the later stage embryos grew or formed callus, but not all the globular or late globular embryos responded (Table 9).

Table 9. Response of Hybrid Embryos Cultured at Different Stages of Development.

Stage of Development of Embryo							
Globular		Late Globular		Heart		Early Cotyledonary	
NC	NG	NC	NG	NC	NG	NC	NG
A. hypogaea cv M13 x *A.* sp. 276233							
6	1(17)	29	6(21)	1	1(100)	5	5(100)
A. hypogaea cv MK 374 x *A.* sp. 276233							
23	4(17)	61	29(48)	1	1(100)	6	6(100)

NC = Number cultured; NG = Number which grew; ( ) = Percentage.

Embryos were therefore segregated according to stage of growth, and developed embryos were cultured on MS with 3% sucrose, 0.01 mg L^{-1} NAA, and 0.1 mg L^{-1} BA. These embryos grew normally. Less-developed embryos were cultured on the same basic medium but with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA to induce callus formation. Callus was subcultured on the same medium, and multiplied to produce large amounts of callus from one cross-pollination. Callus subsequently transferred to MS with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA formed shoot buds. Cultures with shoot buds were transferred to MS with 0.1 mg L^{-1} NAA and 1.0 mg L^{-1} BA, and shoot buds elongated. The resultant shoots were either grafted onto stocks of *A. hypogaea* or *A.* sp. 276233, or supported on filter paper over MS with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} IBA to induce root formation.

Discussion

Treating flowers with GA at the time of pollination was a simple and effective way to increase the frequency of hybrid pegs. Other techniques such as mentor pollen were also effective but were not adopted as a routine treatment, as GA application using the cotton wool method is quick, does not need special skills, apparatus or the care needed in producing mentor pollen or mentor pollen leachate, and can be done by anyone who can emasculate and pollinate groundnuts without appreciably decreasing the number of pollinations. The levels of peg production achieved are entirely

satisfactory for a practical interspecific crossing program, and are of the same order as in intraspecific crosses.

The mode of action of GA in promoting hybrid peg production in *Arachis* is not clear. Peg growth is the result of the formation of a meristem basal to the ovules, and this meristem is not active in hybrid pegs. At the given concentrations GA does not stimulate peg formation unless fertilization has taken place, as unpollinated ovaries do not form pegs when treated with GA; the maximum peg formation achieved was similar to the maximum achieved with intraspecific crosses, and probably relates to the maximum rate of fertilization that is commensurate with the damage to the style during hand emasculation and pollination. GA has been applied as a spray, lanolin paste, or injection to developing fruits to prevent fruit loss in wide crosses in a number of genera (de Nettancourt, 1977). There is no zone of abscission and no comparable shedding of fruit in *Arachis* crosses. The only abscission zone is at the base of the hypanthium and is involved in the shedding of the flower (Pattee and Mohapatra, 1986). This zone is adjacent to where the meristem develops, and it is possible that GA provides a stimulus to overcome the quiescence of the meristems that is lacking in hybrids.

The auxins IAA and NAA were the most effective of the hormones tested for pod formation, though Kn also had an effect. IAA applied in lanolin, at either 50 or 100 ppm, 20 or 25 days after pollination was the most effective treatment, although there were differences between the four genotypes used.

A single treatment with auxin was equal to or better than multiple treatments in the *Arachis* crosses tested, though in other intersectional or intergeneric crosses multiple treatments, or mixtures of hormones (Attman, 1988), may be needed to maintain fruit development. The action of auxin may be to inhibit further peg elongation, as occurs in *A. hypogaea*, and to initiate pod formation, which is normally initiated by auxins produced by the ovary (Jacobs, 1951).

Enhancement of peg and pod production by hormone treatments did not result in hybrid seed. The pegs and pods grew slowly, and when harvested 60 days after pollination, they contained immature ovules at a time when pods resulting from self-pollination of the maternal parent would normally have been fully mature. In addition to the fact that the hybrid tissue grew very slowly, the growth of the maternal tissue was also much slower than it would have been after selfing.

Even when the heart-shaped or early cotyledonary embryos had been isolated from the maternal tissue and cultured on a range of media, growth was still slow, and the seedlings grew slowly when transferred to soil. Some of the callus which was produced grew rapidly, and the frequency of shoot formation on regenerative callus was comparable to that on similar *A. hypogaea* callus. Grafts however, did not produce flowers, and only 17 percent of grafts survived for more than 4 months (ICRISAT, 1987), though many other interspecific hybrids in *Arachis* are vegetatively vigorous and flower freely. Flowers were produced by a hybrid plant similar to those described in this paper, except that they were derived from a seed which did mature from a hormone-aided cross between *A. monticola* (a close wild relative considered by some to be a subspecies of *A. hypogaea*) and *A.* sp. 276233.

Conclusions

The need for two hormone treatments to maintain pod growth, and for ovule and embryo culture to maintain the growth of the hybrid zygote, along with the slow growth and lack of flowering of the hybrid plant, indicate that section *Rhizomatosae* has diverged considerably from *A. hypogaea*. The barrier to hybridization apparently is not a simple one. However, the production of hybrids indicates that there is potential for introgression from *Rhizomatosae*, and the techniques can be used to produce hybrids from other intersectional crosses.

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