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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.