1248

Reprinted from

NEW GENETICAL APPROACHES TO CROP IMPROVEMENT

Edited by

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PIDC PRINTING PRESS (PVT)LTD.KARACHI

(1986)

Wide hybridization in *Arachis*: problems and prospects

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Summary

The genus *Arachis* includes a large number of species including *A. hypogaea*, the groundnut. The species are grouped into seven sections. *A. hypogaea*, a tetraploid, can be crossed only with the species within its own section, *Arachis*, even though most of them are diploids. From the diploid species resistances are being transferred to *A. hypogaea*. Investigations on barriers to hybridization between sections have been initiated at ICRISAT. Some techniques have been developed to break the barriers.

After incompatible pollinations between *A. hypogaea* or *A. monticola* and species in section *Rhizomatosae*, fluorescence microscopic investigation reveals that some inhibition of pollen tube growth occurs in the style. However, pollen tubes can be seen at the base of the pistils. In very few instances pegs do develop but do not elongate further than 1 cm and degenerate before they can penetrate the soil and form pods.

Applications of growth hormones to the bases of incompatibly pollinated flowers just after pollination significantly increased the number of pegs produced from about 10 percent (untreated) to more than 50 percent (hormone treated) of pollinations. Hormones were applied singly or in combination, or in sequence. GA3 induced most pegs, but they died before pods were formed. A subsequent treatment with kinetin maintained peg growth to a stage at which embryos could be dissected from immature pods and successfully cultured *in vitro*

Introduction

The genus *Arachis* includes a large number of species, including *A. hypogaea*, the cultivated groundnut or peanut. Many of the wild species are potential sources of genes for improvement of *A. hypogaea*, especially genes for disease resistance (Moss, 1980).

The genus has been divided into seven sections, and hybrids have been produced between *A. hypogaea* and all species in the same section, *Arachis* (Gregory and Gregory, 1979). Disease resistance and other desirable characters from some of these species have been transferred to

A. hypogaea (Moss, 1980 ; Marfo, 1981 ; Singh *et al.*, 1980). Attention has been focussed on the section *Rhizomatosae* which contains a number of species which are resistant to pests and diseases, including resistance to some viruses (Moss, 1980). However, most attempts to produce hybrids between these species and *A. hypogaea*, have been unsuccessful, and very few hybrids have been produced by conventional means (Gregory and Gregory, 1979).

Fruit formation in all species in the genus is complex. The sepals, petals, stamens and stigma are borne on a hypanthium, with the ovary at the base of the hypanthium. The hypanthium, sepals, petals and stigma die within a few days after anthesis, but if fertilization occurs, an intercalary meristem basal to the ovules becomes active, and a gynophore (or 'peg') is formed, which elongates and carries the ovary at its tip into the soil where the fruit is formed.

After incompatible pollinations, a range of reactions has been observed, from a complete lack of visible response, death of partially developed pegs to production of seedless pods. Many techniques are possible to overcome these reactions (Figure 1).

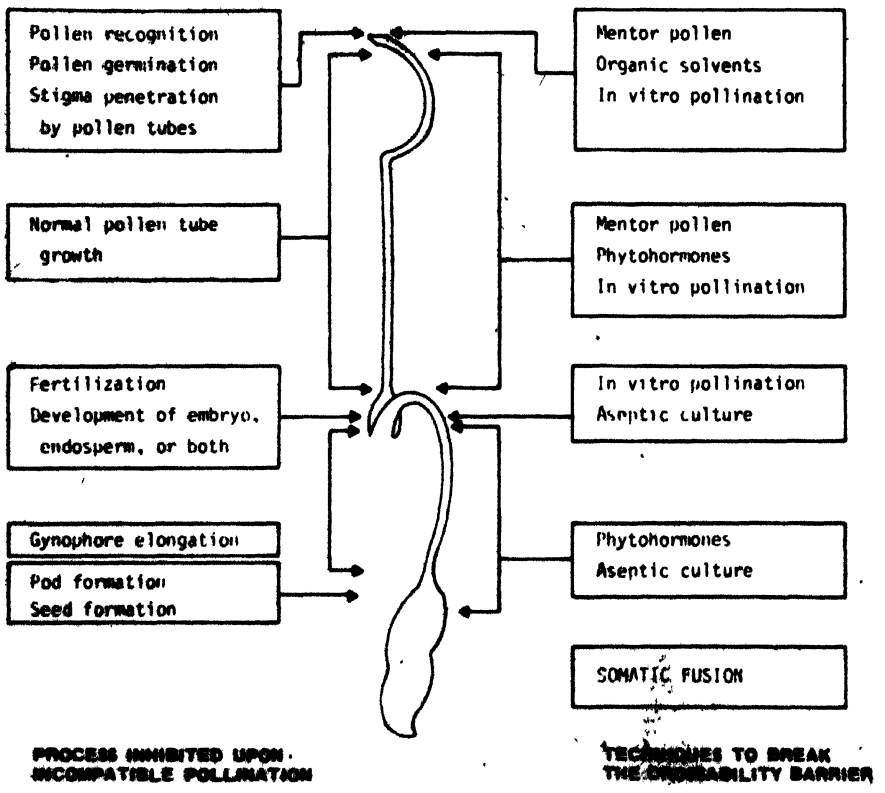

Figure 1. Fruit development and incompatibility in *Arachis*.

This paper reports our attempts to produce large numbers of culturable embryos from crosses between *A. hypogaea* (and its closest relative *A. monticola*) and species in the section *Rhizomatosae* to facilitate gene transfer to the cultivated species. The techniques for culture of embryos have been developed (Sastri *et al.*, 1980, 1981) and some hybrids have been produced (Sastri and Moss, 1981).

Materials and methods

The taxa used in the present investigations are shown in Table 1. All the species used are tetraploids (2n=4x=40).

Plants were grown in insect-free conditions to prevent cross pollination. Young buds of female parents were emasculated the day before anthesis, and pollinated the following morning.

Table 1. Source and accession data of species used

Parents	Species	ICG No.	P. I. No.	Collector Initial* and No.	Source
Female Parents	*Arachis hypogaea* cv. Robut 33-1	799	—		Kadiri, A. P., India
(in Section) (*ARACHIS*)	*Arachis monticola*		263393/298364-5 331338-9	8135 HLP 104	219824 North Carolina State University, U.S.A.
Male Parents	*A. glabrata*	8902			Tamil Nadu Agriucltural University, Coimbatore, India
(in Section) (*RHIZOMATOSAE*)	*Arachis* sp.	8176	276233	GK 10596	Reading Univ., U.K.
	Arachis sp.	8165	262844	GKP 9649	Reading Univ., U.K.

*G=Gregory H=Hammons K=Krapovickas L=Langford P=Pietrarelli

For the study of pollen germination and pollen tube growth, pistils pollinated with compatible (self) pollen or with incompatible *Rhizomatosae* pollen were fixed in acetic acid : ethanol (1 : 3) 24h after pollination, cleared overnight in 8N NaOH and stained with decolourised aniline blue for observation by fluorescence microscopy.

Bud pollinations were done 2 days and 1 day before anthesis, and delayed pollinations one day after anthesis.

Mentor pollen, which had been prepared by treating compatible pollen with anhydrous methanol for 1 min, and evaporating the methanol,

was mixed with an equal volume of incompatible pollen. Mentor pollen leachate was prepared by suspending compatible pollen from 10 flowers in 1 ml of 0.025M Tris-HCl buffer with 0.01M $CaCl_2.2H_2O$ with 10% sucrose, for 45 min at 4°C. The suspension was filtered, and the filtrate was applied to the stigma by camel hair brush immediately before incompatible pollinations.

The phytohormones used were gibberellic acid (GA3), indole acetic acid (IAA), naphthalene acetic acid (NAA), kinetin (Kn) and benzyl amino purine (BAP).

Three concentrations (5.00×10^{-5}M, 1.25×10^{-4}M and 2.50×10^{-4} of two auxins, IAA and NAA were used. The crosses investigated were *A. monticola* × *Arachis* sp. P. I. No. 276233 and *A. hypogaea* cv. Robut 33-1 × *Arachis* sp. P. I. No. 276233.

Four concentrations (2.50×10^{-4}M, 1.25×10^{-4}M, 6.25×10^{-5}M and 3.13×10^{-5}M) of GA3 were applied to flowers of *A. monticola* pollinated with *Arachis* sp. P. I. No. 276233. Pistils were also treated with more than one hormone. The following treatments were examined :

(a) a single treatment : a mixture of cytokinin (Kn) of auxin (IAA or NAA) with gibberellic acid; the mixture of hormones was prepared in such a way that it contained the hormones at their individually effective concentrations.

(b) multiple treatments : an initial treatment with gibberellic acid followed by one, two, or three treatments with cytokinin (Kn) or auxin (IAA) at certain intervals.

These phytohormones were applied in absorbant cotton wool wrapped around the base of the hypanthium at the time of pollination. Emasculated flowers, untreated and pollinated with incompatible pollen, and unpollinated flowers treated with phytohormones, were used as controls. The techniques of culturing hybrid embryos formed following the above treatments have been described by Sastri *et al.* (1980 and 1981).

Results

ıllen germination and pollen tube growth (untreated) ꟻollinations at anthesis

Pollen tubes in compatible crosses contained uniformly distributed small callose plugs. After incompatible pollinations, pollen germination was satisfactory, but the growth of the tubes in the style was not normal. Tubes varied in diameter and had uneven distribution of large callose plugs. Despite these abnormalities, pollen tubes were seen in the ovary, and the production of a number of pegs and pods with aborted embryos after incompatible pollinations (Tables 3 and 4) indicates that some pollinations were effective.

Table 2. Peg formation in the intersectional cross *A. monticole*×*A. glabrata*

Treatments :	Percent pegs formed
Control (untreated pollen)	0
Mentor Pollen	3
Mentor Pollen Leachate	22
Mentor Pollen Leachate+Kinetin 10^{-6}M	53
Kinetin 10^{-6}M	15

Table 3. Effect of two auxins on peg and pod formation in an incompatible cross *A. hypogaea cv Robut 33-1*×*A. sp.* P. I. No. 276233

Concentration	I A A			N A A		
	No. of Pollina-tions	Percent pegs formed	Percent pods formed	No. of Pollina-tions	Percent pegs formed	Percent pods formed
5.00 × 10^{-5}M	95	8	2	52	23	15
1.25 × 10^{-4}M	102	10	1	75	24	8
2.50 × 10^{-4}M	100	5	2	52	4	4
Control*	152	15	5	152	15	5

* Pollinated, no hormone treatment

Table 4. Effect of two auxins on peg and pod numbers in an incompatible intersectional cross *A. monticole* × *A. sp.* P. I. No. 276233

Concentration	I A A			N A A		
	No. of Pollina-tions	Percent pegs formed	Percent pods formed	No. of Pollina-tions	Percent pegs formed	Percent pods formed
5.00 × 10^{-5}M	82	6	0	98	7	0
1.25 × 10^{-4}M	134	9	0	138	15	0
2.50 × 10^{-4}M	80	10	1	110	14	1
Control*	364	16	1	364	16	1

*Pollinated, no hormone treatment.

Early or delayed pollinations

There was no germination of either compatible or incompatible pollen when applied to stigmas either before or after the day of anthesis

Mentor pollen and mentor pollen leachate

Although mentor pollen and mentor pollen leachate were effective in increasing the number of pegs formed (Table 2), the pegs degenerated before penetrating the soil, and no pods were formed.

Peg and pod formation

No pegs were produced by controls (emasculated, unpollinated flowers treated with hormones).

Investigations on cross incompatibility between tetraploid species of the section *Arachis* and the section *Rhizomatosae* revealed that after untreated pollinations pegs were formed in fewer than 20% of the pollinations but none developed into pods (Anon, 1981). Application of gibberellic acid (GA3), kinetin (Kn) or naphthyl acetic acid (NAA) not only stimulated the production of more pegs, but also sustained their subsequent growth to a stage when pods were formed.

Effect of auxins

At the concentrations used, IAA was not effective in inducing more pegs than in the pollinated untreated controls in both the crosses (Tables 3, 4). Two concentrations of NAA ($5.00\times10M^{-3}$, 1.25×10^{-4}M) produced more pegs in *A. hypogaea* only, and many of these formed pods also (Table 3). However, they were not effective in the cross *A. monticola*, × *A.* sp. P. I. No. 276233 (Table 4).

Effect of gibberellic acid

Even at the lowest concentration (3.13×10^{-5}M), GA3 was found to stimulate pegs in 56% of pollinations in *A. monticola* crosses (Table 5). With increase in GA3 concentration the peg numbers increased, reaching 87% of the total pollinations. Subsequently, a few pods were obtained from these pegs but there was no consistency in this response, and the

pods developed rather slowly and rarely matured fully. GA3 at 2.50×10^{-4}M was tried in *A. hypogaea* crosses with 3 other incompatible male parents and was found to be effective for pod production, though the number of embryos which could be cultured varied (Table 6).

Table 5. Effect of gibberellic acid on peg and pod numbers in an incompatible cross *A. monticola* × *A. sp.* P. I. No. 276233

GA concentration	No. of pollinations	No. of pegs formed
3.13×10^{-5}M	27	14 (56%)
6.25×10^{-5}M	26	19 (73%)
1.25×10^{-4}M	21	18 (87%)
2.50×10^{-4}M	45	36 (80%)
Control (No GA3)	364	57 (16%)

Table 6. Effect of GA (2.50×10^{-4}M) on *A. hypogaea* cv. *Robut 33-1* pollinated with 3 accessions of the section *Rhizomatosae*

Male Parent	No. of pollinations	Percent pegs	Percent pods	Percent embryos
P. I. No. 276233	262	51	13	9
Coll. No. 9649	54	59	20	2
A. glabrata	55	60	13	4

fect of cytokinins

Two cytokinins (kinetin and BAP) were previously investigated at three or four concentrations for their ability to induce peg and pod formation in incompatible crosses (Anon 1981). It was found that both the cytokinins at certain concentrations (10^{-7} M,10^{-6}M, 10^{-5}M) stimulated peg initiation and elongation in both crosses, but their most significant effect was to stimulate pod and seed development. Pegs formed after kinetin treatment were able to penetrate the soil but untreated pegs died before this stage. However, kinetin induced fewer pegs than GA3, but induced 51 pods per hundred pollinations.

Subsequent experiments have therefore been conducted to exam the combined effects of GA3, kinetin and auxins.

Effect of combinations of hormones

Single treatment with hormone mixtures

In *A. hypogaea* crosses, kinetin (10^{-7}M) mixed with GA3 (2.50×10^{-4}M) produced fewer pegs than GA3 alone (Table 7). Bu this mixture had the advantage that the presence of kinetin stimulatec a greater proportion of pegs to form pods from which embryos were obtained which could be successfully cultured. This was also the case with the other cross *A. monticola* × *Arachis* sp. P. I. No. 276233 (Table 8). These observations indicate that kinetin plays a role in pod, seed anc embryo development.

A mixture of kinetin (10^{-7}M) and NAA (1.25×10^{-4}M) was not beneficial in *A. hypogaea* and *A. monticola* crosses (Tables 7, 8).

Table 7. Effect of hormones and their combinations on peg and pod numbers in an incompatible cross, *A. hypogaea cv. Robut 33-1* × *Arachis sp.* P. I. No. 276233

Treatment	No of pollinations	Percent pegs formed	Percent pods formed	%Pods per peg	Percent embryos cultured
Kn 10^{-7}M + GA 2.50×10^{-4}M	102	16	5	31	5*
Kn 10^{-7}M + NAA 1.25×10^{-4}M	76	1	0	0	0
GA 2.50×10^{-4}M	262	51	13	25	9**

*Embryos 0.2 to 0.5 mm, 2 embryos grew when cultured

**Embryos 0.1 to 0.2 mm, none grew when cultured

Table 8. Effect of hormones and hormone mixtures on peg and pod numbers in an incompatible cross. *A. monticola* × *Arachis sp.* P. I. No. 276233

Treatment	No. of pollinations	Percent pegs formed	Percent pods formed	No. of embryos cultured
Kn 10^{-7}M + GA 2.50×10^{-4}M	280	45	6	1 (mature)
Kn 10^{-7}M + NAA 1.25×10^{-4}M	92	20	0	0
GA 2.50×10^{-4}M	142	51	4	0

Multiple treatments

Gibberellic acid (2.50×10^{-4}M) was used as an initial treatment at pollination because of its ability to produce pegs in large numbers (Table 5). In both the crosses *A. hypogaea* × *Arachis* sp. P. I. No. 276233 and *A. monticola* × *Arachis* sp. P. I. No. 276233, the GA3 treatment induced pegs in most pollinations, up to 100% in many cases. Most of the pegs were already formed by the time later treatments were administered.

The number of pegs producing pods due to an initial GA3 treatment followed by one or more kinetin treatments varied from none to 100% depending upon the treatment. In some cases the embryos could be dissected and cultured. Among these treatments kinetin gave the largest number of dissectable embryos per pollination when applied on the 25th day after pollination, followed closely by the treatment where kinetin was applied three times on the 10th, 17th and 22nd days after pollination (Table 9).

When GA3 treatment was followed by one or more IAA treatments at 2.50×10^{-4}M, the number of pegs forming pods was less than that formed after kinetin treatment in both the crosses. In *A. hypogaea* crosses maximum embryos per pod, per peg and per pollination were obtained after IAA treatment on either the 10th or 15th day after pollination (Table 9).

Table 9. Percent embryos cultured in *A. hypogaea* × *Arachis sp.* P. I. No. 276233. Pegs were induced by GA3 at pollination and subsequently treated with Kn or IAA

Time of subsequent treatment(s) (days after pollination)	Kn(10^{-7}M)		IAA (2.5×10^{-4}M)	
	No. of polls	Per cent embryos	No. of polls	Percent embryos
10	35	30	38	34
10, 17	22	14	16	0
10, 17, 22	15	47	21	14
15	35	23	18	39
15, 22	13	15	19	5
15, 22, 29	13	8	14	0
20	9	23	20	10
20, 25	—	—	25	0
25	11	55	—	—

Discussion

The results show that it is possible to stimulate peg and pod formation in incompatible crosses in *Arachis* to a stage when embryos can be cultured from crosses from which hybrids have not been previously obtained. The application of hormones in cotton wool at the time of pollination is a simple technique that does not require sophisticated equipment, and allows many pollinations to be made with the possibility of producing hybrids in large numbers. The other techniques tried, using killed but chemically active mentor pollen and mentor pollen leachate, were more laborious, and restricted the number of pollinations that were possible.

The results also indicate that the development of the ovary into a pod is regulated by hormones. The processes involved are complex : peg initiation and elongation, during which the embryo ceases division, is followed by pod formation with the resumed growth of the embryo (Smith, 1956). These processes are probably under the control of a number of hormones, as indicated by the greater number of culturable embryos obtained by consecutive treatments with different hormones. Elucidation of the hormone requirements at different stages of development would permit formation of mature seed on the plant after intersectional pollinations. Until then, the undeveloped or partially developed embryos can be cultured for the production of hybrids.

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Discussion

Schlichting E. (Germany): The figures you gave for the water retention capacity of Vertisols have obviously been determined with swollen samples. From the ecological viewpoint, such figures are misleading since the pore size distribution varies with soil moisture, i.e. the permanent wilting point (PWP) and the field capacity (FC) are not constants. Vertisols may supply plants with water while the average soil moisture is below the PWP and may have seepage while this moisture is below FC, depending on the degree of cracking before rain or irrigation and on the rain density (mm/h). Do you have data on the pore size distribution (water retention) of Vertisols in different moisture status?

Answer: I do not have such data. In the case of Vertisols with deep profile, there is plenty of water available in deeper layers but not on the surface. We have introduced the system of dry sowing because of our awareness of changes in the pore size distribution.

Kyuma, K (Japan): Please tell me what "vertical" mulching is.

Answer: This practice consists of burying sorghum stubbles and straw in trenches 40 cm deep, 15 cm wide and protruding 10 cm above the ground level. Such practice enables to increase water intake and moisture storage in deep Vertisols of independable rainfall areas (about 500 mm). There is a reference on this method in my paper.

Kubota, T. (Japan): You mentioned that Vertisols were not prone to crust formation or erosion. On the other hand, slaking properties after drying (large clods to fine aggregates) are conspicuous, resulting in the packing of large pores on the soil surface, which in turn is detrimental to crop growth. Have you observed such phenomena?

Answer: I have not observed such phenomena as the Vertisols I referred to in my presentation occur on a 3% slope in a low rainfall area. The Vertisols you referred to in Thailand are located in flat areas and slaking may be due to internal drainage. In India, Vertisols do not present much of a problem for sorghum or millet crops whereas Alfisols under the same climatic conditions pose a serious problem of crusting which affects germination and crop stand establishment.

Briones, A.A. (The Philippines): At what moisture content for the type of Vertisols you have shown do you observe the cracks? How much shrinkage does occur?

Answer: The total amount of water retained in the profile may be 800 to 900 mm, out of it the available moisture must range between 200 and 300 mm. Shrinkage occurs at less than 10%, probably in relation to changes in the pore size distribution.