Rooting in Leaf Petioles of *Arachis* for Cytological Analysis

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Summary Method of rooting in petioles was developed for leaves of *Arachis* plants. The objective was the development of methodology that allows the chromosome number determination for evaluation of the ploidy level in colchicine-treated branches. In order to obtain a high frequency of metaphases and chromosomes with clear morphology, roots were pretreated with 8-hydroxyquinoline combined to cycloheximide for 2 h. Root tips were stained using Feulgen dye for chromosome analysis. This method can be applied also for cytological studies of materials in which seeds are not available or cutting is not feasible.

Key words *Arachis*, Chromosomes, Rooting, Polyploidy, Cytology.

Wild *Arachis* species have been extensively studied due to their potential use in groundnut (*Arachis hypogaea* L.) breeding and as forage and ornamental crops. Several wild species have shown interesting characteristics for groundnut breeding programs, mainly involving biotic resistance factors (Gardner and Stalker 1983, Stalker and Moss 1987, Stalker 1989, Starr *et al.* 1990, Singh *et al.* 1996, Simpson and Starr 2001).

Arachis hypogaea is an allotetraploid (AABB) (Singh *et al.* 1991, Krapovickas and Gregory 1994), with $2n=40$. Most of the wild *Arachis* species are diploid with $2n=20$ (Fernández and Krapovickas 1994). In *Arachis* section, several wild species have A genome, which can be cytologically characterized and differentiated from other species by the presence of a pair of chromosomes much smaller than the others, with different staining characteristics (Husted 1933, 1936). It was believed that there was another genome in groundnut, which did not have the pair “A”, and which could be observed with a secondary constriction and an associated satellite in one chromosome. These chromosomes were called “chromosomes B” in classical works (Husted 1936). Presently, however, it is known that all species cytologically analyzed in the genus *Arachis* have a chromosome pair (and eventually two) with satellites, called chromosome SAT (Fernández and Krapovickas 1994). Thus the presence of a SAT chromosome does not guarantee the exact classification as a representing of the B genome of the groundnut. Consequently, the general concept of B genome species is more restricted, requiring being diploid and belonging to the *Arachis* section, sharing the same B genome of the cultivated groundnut and not having the pair “A”. Thus there is no guarantee that all species of *Arachis* Section that do not have “A” pair shares the same B genome of the cultivated groundnut. In this paper, such species are referred to as presenting a “non-A” genome. This includes those diploids species belonging to the *Arachis* section, but not having the “A” pair and not necessarily sharing the same B genome of the cultivated peanut.

The objective of this paper was to present a method to obtain rooting for cytological analysis and to adapt a root pre-treatment and chromosome preparation protocol for analysis of somatic chromosomes of *Arachis*.

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Materials and methods

Ten accessions of wild species with A genome ($2x=20$) were crossed to five accessions of wild species with “non-A” genome ($2x=20$) generating sterile F_1 individuals, which were treated with colchicine for chromosome duplication.

Some techniques were considered to obtain a preliminary evaluation of the ploidy level of the F_1 's individuals cuttings treated with colchicine. Data referring to the viability of pollen, size and number of stomata for each area unit can indicate parameters of different ploidy levels among the genotypes (Barbosa and Vieira 1997, Tallury and Copeland 2002). However, most of the cuttings did not produce flowers that could be used to test pollen viability, besides the fact that tetraploidized plants tend to retard the period of offspring when compared to diploid hybrids (C. E. Simpson, personal communication). A technique for determining stomata number and size has not been conclusive. Thus, to verify the ploidy level, a technique for direct chromosomes counting from colchicine-treated branches was tried.

The experiment was carried out at Piracicaba-SP, Brazil ($22^{\circ}42'S$ and $47^{\circ}38'W$) under greenhouse condition, with the temperature varying from 21 to $38^{\circ}C$. New totally expanded leaves from colchicine-treated cuttings had their petioles cut and sprayed with the rooting hormone indol-butiric acid (IBA) in their extremities and immediately put in plastic cups with vegetable subtract. Cups were put on a tray and covered with a transparent plastic bag for maintenance of humidity. Seventeen interspecific hybrids combinations were analyzed (Table 1).

Methodologies of root treatment and cytological preparations were adapted from Aguiar-Perecin and Vosa (1985) and Silvarolla and Aguiar-Perecin (1994). Inhibitor of the mitotic spindle fibers (8-hydroxyquinoline) and the protein synthesis inhibitor (cycloheximide) were used for optimizing chromosome preparations having spread chromosomes, clear morphology and high metaphase frequency. Roots with approximately 1 cm were collected, immediately treated with a solution of 300 ppm 8-hydroxyquinoline and 3.125 or 6.25 ppm cycloheximide during 1.5 h and 2 h respectively, and fixed with ethanol/acetic acid (3:1). Roots were stained according to Feulgen methodology, hydrolyzed in 1 N HCl for 9 min at $60^{\circ}C$ and stained with Schiff's reagent for 45 min,

Table 1. Hybrids, species and respective accessions used as male and female parents and results obtained with cytological analysis (major ploidy level found)

Female Parents (A genome)			Male Parents (B genome)			Ploidy ($x=10$)
Species	Accession	BRA	Species	Accession	BRA	
<i>A. batizocoi</i>	K 9484	013315	× <i>A. cardenasii</i>	GKP 10017	013404	4x
			× <i>A. duranensis</i>	VNvEv 14167	036200	4x
			× <i>A. helodes</i>	VSGr 6325	012505	2x
			× <i>A. kempff-mercadoi</i>	V 13250	030643	4x
<i>A. magna</i>	VSPmSv 13751	033812	× <i>A. cardenasii</i>	GKP 10017	013404	4x
			× <i>A. stenosperma</i>	Lm 3	036005	4x
			× <i>A. linearifolia</i>	VPoBi 9401	022608	2x
			× <i>A. duranensis</i>	VNvEv 14167	036200	2x
<i>A. aff. magna</i>	VSGr 6389	012696	× <i>A. stenosperma</i>	VGoRoSv 12488	030651	4x
			× <i>A. villosa</i>	VGoMrOv 12812	030813	4x
			× <i>A. kuhlmannii</i>	VSPmSv 13721	033723	4x
			× <i>A. linearifolia</i>	VPoBi 9401	022608	4x
			× <i>A. simpsonii</i>	VSPmSv 13710	033685	2x
<i>A. hoehnei</i>	KG 30006	036226	× <i>A. helodes</i>	VSGr 6325	012505	4x
			× <i>A. cardenasii</i>	GKP 10017	013404	4x
			× <i>A. duranensis</i>	VNvEv 14167	036200	4x
<i>A. ipaensis</i>	KGPScS 30076	036234	× <i>A. villosa</i>	VGoMrOv 12812	030813	4x

in the dark. After the reaction, an enzymatic digestion with a solution containing 2% cellulase and 3% pectinase (both from *Aspergillus niger*) for 10 min at 37°C, succeeded by a washing in citrate buffer (4 mM citric acid+6 mM trisodium citrate) has proceed. For chromosome preparations, the stained meristematic regions were squashed on to a drop of 1% aceto-carmin with a slight compression for chromosome spreading. After selection, coverslips were removed in 45% acetic acid and mounted with Canadian balsam. Photomicrographs of the chromosomes were obtained with a Zeiss photomicroscope, using a Kodak Technical Pan (ISO 25) film.

Results and discussion

The use of detached leaves showed high efficiency for obtaining rooting in petioles. The rooting occurred from one week up to 20 d after the isolation of the leaf. The period of rooting varied, apparently due to the size and age of the leaf. Younger and larger leaves required shorter time for rooting. It is believed that the environment temperature between 21 and 38°C also helped to obtain roots. This rooting technique possibly could be used with other species of plants, like soybean (*Glycine max*), in which the rooting of petioles has also been tested with success by the first author (data not shown). The present technique can also be adapted in case of species with non-availability of seeds and/or impossibility of cutting. The quantity and quality of the roots produced were compatible with the application of cytological techniques. Fig. 1 shows a petiole rooted from a leaf of a hybrid between *Arachis batizocoi* and *Arachis cardenasii*.

The use of a combination of 300 ppm 8-hydroxyquinoline and 6.25 ppm cycloheximide for 2 h was the most efficient treatment and allowed obtaining of cytological preparations with a high rate of metaphases and prometaphases, with an adequate condensation of chromosome, that made it possible their counting and identification in the several hybrid combinations analyzed. Other substances have been already used successfully in the observation of mitotic chromosomes in species of *Arachis*, like paradichlorobenzene (Stalker *et al.* 1991, Stalker and Dalmacio 1981), bromonaphtalene (Peñaloza 1995, Fávero 1999), pure 8-hydroxyquinoline (Fernández and Krapovickas 1994). The satisfactory spreading of chromosomes in the cells can be due to the use of cellulase and pectinase, and to the heating of the slide before the superposition of the coverslips. Various roots of each plant were evaluated. Somatic chromosomes of diploid hybrid ($2x=20$) K 9484 (*A. batizocoi*) $\times$ V 6325 (*A. helodes*) can be visualized in Fig. 2a. Cell of a hybrid with successful duplication of the chromosome number ($4x=40$), resulting from the crossing V 6389 (*A. aff. magna*) $\times$ V 12812 (*A. villosa*) is shown in Fig. 2b. It is important to report the presence of a heteromorphic pair of chromosomes, indicated by arrows in Fig. 2. The presence of only one chromosome "A" was observed, in diploid hybrid individuals, once the crossing always involved accessions with

Fig. 1. Rooting leaf of hybrid between *Arachis batizocoi* (K 9484) and *Arachis cardenasii* (GKP 10017).

Fig. 2. A) Somatic chromosomes of diploid hybrid ($2n=2x=20$) between K 9484 (*A. batizocoi*) $\times$ V 6325 (*A. helodes*), B) Tetraploid hybrid ($2n=4x=40$) between V 6389 (*A. gregoryi*) $\times$ V 12812 (*A. villosa*). Black arrows show “A” chromosomes. Bar=5 μ m.

“A” and “non-A” genomes. In tetraploid individuals duplicated with colchicine a pair of “A” chromosomes was observed, indicating the allotetraploid structure of the individual.

This methodology allowed to recognize the polyploid and the non-polyploid hybrids, as well as counting and clear observation of chromosome morphology. Root pretreatment with combination of hydroxyquinoline and cycloheximide has been successfully used in cytological analysis of several species of plants, such as sugar cane (Silvarolla and Aguiar-Perecin 1994), corn (Bertão and Aguiar-Perecin 2002), *Crotalaria* sp. and *Passiflora* sp. (Cuco *et al.* in press).

Arachis has several wild species with characteristics of low prolificity, and consequently, a small number of individuals in the germplasm banks. Therefore, the cytogenetic characterization through mitotic analysis is much more difficult. The present technique of rooting leaves and the new protocol for root pretreatment may be interesting for species that have the capability of rooting in their petioles.

Procedures described in this paper were applied successfully for the discrimination of artificial polyploid hybrids produced with colchicine treatment, with the purpose of introgression of genes for resistance to *Cercosporidium personatum*, *Cercospora arachidicola* and *Puccinia arachidis* existing in wild species to the cultivated peanut.

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