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Short communication

Culture vessel and auxin treatments affect *in vitro* rooting and *ex vitro* survival of six *Arachis paraguariensis* genotypes

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ABSTRACT

Arachis paraguariensis Chodat and Hassl. is a wild peanut with very high morphogenic potential but limited rooting ability *in vitro* has hampered production of large number of surviving plantlets. The present study evaluated *in vitro* rooting and *ex vitro* survival of micro-shoots utilizing a $6 \times 3 \times 3 \times 2$ factorial experiment. Six *A. paraguariensis* genotypes were treated with three auxins at three concentration levels inside either 11.4 cm × 8.6 cm × 10.2 cm polyethylene terephthalate glycol (PETG) vessel or 2.5 cm × 15 cm glass tube. The auxin treatments included indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA) at 0.2, 0.6 and 1 mg L^{-1}. Across all auxin treatments, PETG vessel was superior to glass tube in terms of rooting percentage (68% *vs* 17%), and survival at acclimatization (38% *vs* 6%) respectively. Further analysis of plantlets cultured inside PETG vessel showed that rooting and post acclimatization survival were high on MS medium without any auxin (90% and 80%) and on 0.2 mg L^{-1} IBA (92% and 76%) or IAA (86% and 74%) respectively. Contrariwise, none of the shoots treated with 1 mg L^{-1} NAA survived acclimatization. The 0.2 mg L^{-1} of IAA and IBA treatments also exhibited high number of roots per plantlet (7 and 5), and lowest number of days to root initiation (11 and 12 days) respectively. Since rooting was delayed (26 days) on MS medium without auxin, we recommend supplementation of nutrient medium with 0.2 mg L^{-1} IBA or IAA whenever rapid rooting is desired. Overall, the results showed that genotype, auxin type, and auxin concentration significantly altered *in vitro* rooting of *A. paraguariensis* plantlets.

1. Introduction

Several wild *Arachis* are important sources of novel genes and therefore possess the potential for use in genetic improvement of their domesticated relatives. *Arachis paraguariensis* (family Fabaceae, subfamily Papilionoideae, tribe Dalbergieae, section Erectoides), a long-lived perennial wild species is of significant importance due to the wide genetic and physiological variations between and within several of its accessions (Krapovickas and Gregory, 2007). Several of these accessions also possess genes for resistance to various diseases including early leaf spot (Subrahmanyam et al., 1995), root-knot nematode (Sharma et al., 1999), and tobacco armyworm (Stevenson et al., 1993). Tissue culture is vital for biotechnological advancement and genetic conservation of plant species, hence, the importance of being able to successfully regenerate *A. paraguariensis in vitro* from cell, tissue and organs cannot be overemphasized.

The high morphogenic potential of this species has been confirmed by several studies (Pacheco et al., 2007; Aina et al., 2012; Li et al., 1993; Still et al., 1987), hence, its suitability as a model system for studying tissue regeneration and morphogenesis in leguminous species. But despite these potentials, problematic rooting *in vitro* and *ex vitro* have hindered production of large number of surviving plantlets (Li et al., 1993; Still et al., 1987). Limited rooting ability in tissue cultured derived plants is a significant factor challenging the genetic improvement and conservation of several species (Bairu and Kane, 2011; Cati et al., 2014; Vahdati et al., 2004) because the root system takes part in many signaling pathways that determine *ex vitro* acclimatization and subsequent plantlet survival.

Controlling physical and chemical environments of tissue culture explants can improve morphological characteristics of plantlets and their acclimatization to *ex vitro* conditions (Majada et al., 2000; Lucchesini and Mensuali-Sodi, 2004; Huang and Chen,

Abbreviations: IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; BA, 6-benzylaminopurine; MS, Murashige and Skoog basal medium; NAA, α-naphthalene acetic acid; PETG, polyethylene terephthalate glycol; TDZ, thidiazuron.

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2005; Liu et al., 2013). Factors such as medium composition, light, temperature, auxin, culture vessel type, and the air velocity in the growth chamber have been known to influence *in vitro* rooting in *Arabidopsis* (Rolli et al., 2012; Kakani et al., 2009), apricot (Cati et al., 2014), sorghum (Liu et al., 2013), and *Phillyrea latifolia* (Lucchesini and Mensuali-Sodi, 2004). Different forms of auxins such as α-naphtaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) have been used to promote *in vitro* rooting in *A. paraguariensis* (Still et al., 1987; Li et al., 1993). Since rooting rarely occurred in agar or liquid cultures, *in vitro* derived shoot were either grafted to the stem of another species, *A. hypogaea* (Still et al., 1987) or rooted on porous root cubes saturated with modified MS medium containing 1 mg L^{-1} NAA (Li et al., 1993). Although Pacheco et al. (2007) later suggested the use of nutrient medium without growth regulators; no data was presented on *in vitro* rooting frequency and *ex vitro* plantlet survival.

Since *in vitro* rooting of *A. paraguariensis* plantlets has led to unclear results, we propose that this is the critical phase of the entire tissue culture regeneration process. Hence, there is need to create appropriate micro-environmental conditions *in vitro* and *ex vitro* so that root organogenesis and photosynthetic activity may be improved. The present study was carried out to identify the factors influencing *in vitro* rooting in *A. paraguariensis* in order to overcome the challenges that have limited extensive application of *in vitro* techniques for genetic conservation of this species.

2. Materials and methods

2.1. Tissue culture explant

The seeds used for deriving explants were obtained from USDA Plant Genetic Resources Conservation Unit, Griffin Georgia; a component of the Germplasm Resources Information Network (GRIN) National Plant Germplasm System (NPGS). The identity and geographical origin of these genotypes are presented in Table 1. Seeds were manually shelled followed by surface sterilization in 70% ethanol for 1 min and treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min before they were rinsed thoroughly with sterile-distilled water. Soaking in sterile water for 4 h in a laminar airflow chamber was carried out prior to seed coat removal. The seeds were then carefully dissected into four equal longitudinal pieces to derive quartered seed explants.

2.2. Maintenance of mother shoot cultures

In order to develop micro shoots for this study, mother shoot cultures of six genotypes of *A. paraguariensis* were initiated based on the methods of Aina et al. (2012). The media formulations consisted of MS basal salts (Murashige and Skoog, 1962), vitamins as in Gamborg B5 medium (Gamborg et al., 1968), 30 g L^{-1} sucrose (Sigma # S5390), 0.8% (w/v) agar (Sigma # A7921), and supplementation with 2.2 mg L^{-1} thidiazuron (TDZ) and 4.4 mg L^{-1} 6-benzylaminopurine (BA). The pH of each medium was adjusted to 5.8 using 0.1 N KOH prior to autoclaving at 121 °C with 1.06 kg cm^{-2} pressure for 20 min. Liquid medium (25 ml) was dispensed into each 2.5 cm × 10 cm petri dish, and each quartered seed explant was carefully implanted on the medium with its cut ends embedded. The petri dishes which were covered and wrapped with parafilm® were then placed in a growth chamber at 26 ± 1 °C under continuous lighting at 60 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) supplied by cool-white fluorescent lamps.

Table 1
The identity of six genotypes of *A. paraguariensis* used for the *in vitro* rooting study.

NPGS/GRIN accession No	Taxonomic authority	Geographical origin
Grif 15201	*A. paraguariensis* Chodat & Hassl.	Paraguay
Grif 15208	*A. paraguariensis* Chodat & Hassl.	Paraguay
PI 262842	*A. paraguariensis* subsp. *paraguariensis*	Brazil
PI 468155	*A. paraguariensis* subsp. *paraguariensis*	Brazil
PI 468362	*A. paraguariensis* subsp. *paraguariensis*	Paraguay
PI 468365	*A. paraguariensis* subsp. *paraguariensis*	Paraguay

2.3. In vitro rooting of micro-shoots

Micro-shoots (Fig. 1A) of 1–2 cm height were collected from mother cultures after 30 days of culture initiation. These micro shoots were carefully implanted on semi-solid MS medium supplemented with either IAA or IBA or NAA each at concentrations of 0.2, 0.6 and 1 mg L^{-1} inside either 11.4 cm × 8.6 cm × 10.2 cm polyethylene terephthalate glycol (PETG) vessel or 2.5 cm × 15 cm glass tube. Stock solution of each auxin was first filter-sterilized through a double 0.2 μm filter before being added to autoclaved media in sterile bottles. Routinely, 1 ml L^{-1} of Plant Preservative Mixture (PPM™, Plant Cell Technology, Washington DC, USA) was added to the culture medium to prevent contaminations. Each PETG vessel contained four micro-shoots on 50 ml of medium, while only one micro-shoot was plated on 10 ml of medium per glass tube. For every auxin treatment and treatment combination, 8 micro-shoots in 4 replications were evaluated per culture vessel type for each genotype. The vessels and tubes were sealed with Parafilm® before they were incubated at 26 ± 1 °C under continuous lighting provided by cool white fluorescent lamps at 60 μmol m^{-1} s^{-1} PAR.

Data collection on rooted shoots and number of roots were performed after 6-wk of subculture. Afterwards, all surviving plantlets were transferred into Jiffy® peat pellets and acclimatized in a humidity chamber by exposing them to a linear reduction in relative humidity (RH) from 95% to 50% over a period of 2 wk. Hardened plantlets were transplanted into pots containing Metro-mix 300 (Sun Gro Horticulture, Canada Ltd.) and sand in a ratio of 1:1 (v/v). After 4 wk, the surviving plants were counted.

2.4. Experimental design and statistical analysis

This study was a 6 (genotypes) × 3 (auxin types) × 3 (auxin concentrations) × 2 (culture vessel) factorial experiment in a randomized complete block design with 4 replications. Proc. MIXED (SAS Institute, 2010) was used for all data analysis according to a mixed effects model. In order to determine the effects of culture vessel, auxin type, and auxin concentration on rooting and survival of plantlets, each replicate was treated as block. Culture vessel, auxin type and auxin concentration were treated as fixed effects while genotype, replicate and the interactions were considered as random effects. The test for significance of treatment effects was based on ANOVA, while mean separation was performed using the Tukey's Honestly Significant Difference Test ($P = 0.05$).

3. Results

3.1. Root initiation and proliferation

Visual observations during the study revealed that roots of micro-shoots grown on medium containing NAA inside PETG vessels were morphologically different from roots of all other micro-shoots. These roots appeared thicker and short as shown in Fig. 1B. On the other hand, many of the micro-shoots plated on medium enclosed in glass-tubes failed to produce roots but they