

Photoperiod Affects *in Vitro* Flowering in Wild Peanut *Arachis paraguariensis*

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ABSTRACT

Arachis paraguariensis, a wild peanut species, is a potential experimental system for studying the molecular mechanisms of flowering in the genus *Arachis*. The present study was carried out to investigate the effect of photoperiod on *in vitro* reproductive behavior of five genotypes of *A. paraguariensis*. Day-lengths of 12, 16 and 24 h were tested to monitor *in vitro* flowering using growth chambers kept at $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $60\% \pm 5\%$ relative humidity under an illumination of $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Flowering percentage of plantlets ranged from 35% to 93%, 20% to 75%, and 5% to 53% for 12, 16 and 24 h day-lengths, respectively. Genotype PI 262842 displayed the highest frequency of flowering under all the day-length treatments but *in vitro* flower bud initiation was delayed. The highest mean flowering percentage of 65% across all the genotypes for plantlets exposed to 12 h photoperiod is indicative that flowering induction actually occurred. The results presented in this paper provide evidence for photoperiodic flowering response as well as the occurrence of short day-length-enhanced flowering in *A. paraguariensis*.

Keywords: Flower; Tissue Culture; Day-Length; Photoperiod; Peanut; *Arachis*; *In Vitro*

1. Introduction

Several wild *Arachis* species constitute important sources of novel genes for improving cultivated peanut (*Arachis hypogaea* L.). Of significant importance is *Arachis paraguariensis* Chodat & Hassl., a perennial wild relative of the peanut having novel traits including disease [1,2], insect [3], and nematode [4] resistance. Low fruiting efficiency due to asynchronous flowering and pod formation is a persistent problem within the genus *Arachis* that often results in considerable seed-yield losses and low germination rates [5,6]. Although photoperiod sensitivity within the genus *Arachis* is widely reported [7,8], the specific role of environmental factors in the regulation of its reproductive performance is poorly understood. For example, results from field studies focusing on the effects of photoperiod on flowering in cultivated peanut were inconsistent due to differential responses among different cultivars [9,10].

Gaining a proper understanding of the molecular basis for flowering mechanisms should enable the plant breeder to efficiently manipulate reproduction processes for achieving significant impacts on yield and other important traits. *In vitro* flowering, a common phenomenon in several plant species offers an ideal experimental system for studying these mechanisms. Additionally, tissue culture techniques such as *in vitro* fertilization, peg culture

and embryo rescue can be explored as a means of overcoming the long existing hybridization barriers between cultivated peanut and several of its wild relatives.

In vitro tissue explants for plant regeneration are grown on nutrient medium inside confined vessels; therefore, precise control of all environmental factors is expected. Nevertheless, seasonal effects of explant collection and culture initiation on plantlet regeneration have been reported for many species [11-15]. Furthermore, different cytokinins, sucrose concentrations, photoperiod, and subculture time have been used to promote *in vitro* flowering [4,11]. Flowering *in vitro* has been observed in a few *Arachis* spp., however, the photoperiodic flowering response in tissue culture is relatively rare and poorly understood. Still *et al.* [16] and Li *et al.* [17] reported the occurrence of flowering in tissue culture of *A. paraguariensis*, but the environmental and genetic factors involved were not investigated. In this study, a factorial experiment in a completely randomized design with sub-sampling was replicated two times to investigate the influence of photoperiod on *in vitro* flowering of five genotypes of *A. paraguariensis*.

2. Materials and Methods

2.1. Explant Source

Seeds of five genotypes of *A. paraguariensis* used for

this study were obtained from the USDA Plant Genetic Resources Conservation Unit, Griffin Georgia, a division of the Germplasm Resources Information Network (GRIN) National Plant Germplasm System (NPGS). **Table 1** presents the Plant Introduction (PI) numbers and origin of the genotypes. Seeds were shelled manually before storage at 5°C.

2.2. Initiation of Tissue Cultures

Quartered-seed explants of *A. paraguariensis* were derived from whole seeds after surface-sterilization in 70% ethanol for 1 min followed by immersion in 0.1% mercuric chloride for 10 min. The sterilized explants were then rinsed six times in sterile distilled water before they were implanted on MS [18] medium (Sigma # M5524) supplemented with 3% (w/v) sucrose (Sigma # S5390), 0.8% (w/v) agar (Sigma # A7921), 2.2 mg·L⁻¹ thidiazuron (TDZ), and 4.4 mg·L⁻¹ 6-benzylaminopurine (BAP). Vitamins were added according to Gamborg B5 medium [19]. As a routine, 1 mL⁻¹ of Plant Preservative Mixture (PPMTM, Plant Cell Technology, Washington DC, USA) was added to the culture medium before the pH was adjusted to 5.8 using 0.1 N KOH. Medium was autoclaved at 121°C with 1.06 kg·cm⁻² pressure for 20 min.

Stock solutions of TDZ and BAP were sterilized through a double 0.2 µm filter and added to the autoclaved medium. Each 2.5 × 10 cm petri dish containing 4 explants on 25 ml semisolid medium were sealed with Parafilm[®] bands and maintained at 26°C ± 1°C and 60% ± 5% relative humidity under continuous lighting in a growth chamber with illumination of 40 µmol·m⁻²·s⁻¹ provided by daylight-type florescent lamps. After 10 d, regenerated micro-shoots (1 - 2 cm height) were excised from explant-callus masses and sub-cultured into 11.4 cm × 86 cm × 102 cm PhytatrayTM culture vessels (Sigma # P5929) containing 50 ml of MS medium with 3% sucrose (w/v), 0.8% (w/v) agar and no growth regulators. The experiment was a 5 (genotypes) × 3 (photoperiod) factorial experiment in a completely randomized design with sub-sampling, and was replicated twice in time.

2.3. Day Length Treatments

In order to determine the influence of photoperiod on

Table 1. The plant introduction (PI) numbers and origin of five selected genotypes of *A. paraguariensis*.

NPGS/GRIN Accession No	Taxonomic Authority	Origin
Grif 15201	Chodat & Hassl.	Paraguay
Grif 15208	Chodat & Hassl.	Paraguay
PI 262842	Subsp. <i>paraguariensis</i>	Brazil
PI 468362	Subsp. <i>paraguariensis</i>	Paraguay
PI 468365	Subsp. <i>paraguariensis</i>	Paraguay

growth and flowering of *A. paraguariensis* plantlets, day-lengths of 16, 20 and 24 h were tested to monitor *in vitro* flowering in three growth chambers kept at 26°C ± 1°C and 60% ± 5% relative humidity under an illumination of 40 µmol·m⁻²·s⁻¹ provided by cool-white fluorescent light.

For each day length treatment, culture initiation and flowering response were recorded for 10 vessels containing a sub-sample of 4 plantlets each per replicate. From the appearance of the first flower bud until when plantlets ceased to flower, cultures were evaluated in terms of percent flowering, weeks to flowering, and percent peg formation. The plantlets were later transplanted to jiffy pellets and acclimatized before being planted into pots filled with Metro-mix 300 and sand in a ratio of 1:1 (v/v) in the greenhouse.

2.4. Statistical Analysis

Analysis of data was performed according to a mixed effects model using SAS PROC MIXED [20]. Arcsine transformation was carried out on flowering frequency data before analysis while mean separation for days to flower bud initiation was performed using the Tukey's Honestly Significant Difference Test (P = 0.05). Genotype and photoperiod were treated as fixed effects while replicate and the interactions were analyzed as random effects.

3. Results and Discussion

An *in vitro* flowering system is a convenient tool to study specific aspects of flowering, and floral organ development [21]. Certainly, the developmental decision to flower in plants is highly dependent on environmental and endogenous clues. Bünning [22] first proposed that the perception of day-length in plants was influenced by the interaction of light with a circadian rhythm, but innovative studies focusing on flowering in *Arabidopsis* have now provided genetic evidence for the role of the circadian clock in photoperiodic regulation of flowering. The FLOWERING LOCUS T (FT) gene, a major floral inducer in several species, interacts with diverse environmental signals to enable flowering and other developmental processes to be seasonally timed [23-25].

This study was carried out in the years 2009 and 2010 with the aim of investigating the influence of different day-length treatments on *in vitro* flower formation in five selected genotypes of *A. paraguariensis*. Observations from previous experiments [26] had proved that the five genotypes chosen for this study possessed a high frequency of regeneration, rooting and post acclimatization survival. Minimal callus formation was observed on explants after 24 h of culture initiation while shoot bud emergence began as early as 5 d afterwards. Most of the

micro-shoots sub-cultured onto MS medium lacking growth regulators developed rapidly into plantlets within three wk. High frequency flowering was observed for all the genotypes that were studied (**Figure 1(a)**). The *in vitro* flowers appeared normal and were each borne on an erect pedicel as shown in **Figure 1(b)**. Therefore, a pollen germination study was conducted to assess the fertility of *in vitro* derived plantlets and all the pollen samples tested germinated (data not shown). Additionally, further confirmation of a plantlet's fertility was the occurrence of *in vitro* peg formation (**Figure 1(c)**) observed for genotype Grif 15201 while *ex-vitro* seed production (**Figure 1(d)**) was recorded for every plantlets generated across all the genotypes.

A remarkable observation during this study was that most of the plantlets failed to produce flowers during the last quarter of the year, but the regeneration frequency of explants was comparable from one season to another throughout the study. Hence, the scanty data obtained during those months were excluded from the results reported in this paper. It is important to note that the growth chambers used for the experiments were obtained from the same manufacturer and were equipped with digital sensors that offer stringent control over growth conditions for temperature, humidity, light intensity, and photoperiod. Several reports on seasonally dependent tissue culture responses have focused on the period of explant collection and culture initiation [27-29]. The storage duration for the seeds used for deriving the explants in this study varied, thus a possible explanation for the seasonal variation that was observed.

The flowering response of five *A. paraguariensis* genotypes as influenced by day-length treatment is presented in **Table 2**. The mean treatment effects across the genotypes are also displayed. *In vitro* flowering was recorded for all genotypes and every day length. The high-

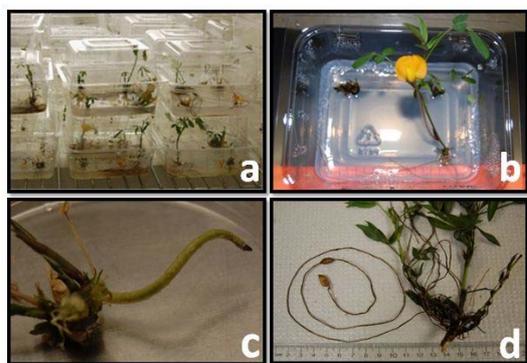


Figure 1. (a) High frequency *in vitro* flowering observed in *A. paraguariensis*; (b) Normal flower borne on an erect pedicel with viable pollen; (c) *In vitro* peg formation observed for genotype Grif 15201; (d) Regenerated plantlets with pod formation after transplanting to pots in the greenhouse.

est flowering percentage of 65% recorded across all the genotypes for plantlets exposed to 12 h day-length is indicative that flowering induction actually occurred. Regression analysis revealed that reducing the day length treatment from 24 to 12 h resulted in a significant linear increase ($P < 0.05$, $R^2 = 0.92$) in flowering frequency across all the genotypes.

Although flowering percentage differed among the five genotypes, all of them, except Grif 15208, showed increased reproductive response under reduced day length. The flowering percentage for 12 h, 16 h and 24 h day length treatment ranged from 35% to 93%, 20% to 75%, and from 5% to 53% respectively. The time taken for plantlets to initiate flower buds across all day lengths is shown in **Figure 2**. Flower bud initiation was relatively delayed in genotype PI 262842 even though this genotype gave the highest percentage of flowering across all the day lengths (**Table 2**). Interestingly, the time taken

Table 2. The effects of photoperiod of *in vitro* flowering in five genotypes of *A. paraguariensis*.

Genotype	Day length (h)		
	12	16	24
Grif 15201	85.0ab*A**	60.0abB	40.5abC
Grif 15208	62.5abA	65.5abA	25.0bcB
PI 262842	92.5aA	75.0aB	52.5aC
PI 468362	50.0bcA	40.0bcB	5.0dC
PI 468365	35.0cA	20.0cB	10.5cdC
Mean	65.0A	52.0B	26.7C
P-value	0.018	0.003	<0.001

*Percentages in the same column for different genotypes followed by the same lower case letter(s) are not significantly different ($P < 0.05$) when subjected to Tukey's honestly significant difference (HSD) test. **Percentages and means in the same row for different day lengths followed by the same upper case letter are not significantly different ($P < 0.05$) when subjected to Tukey's HSD test.

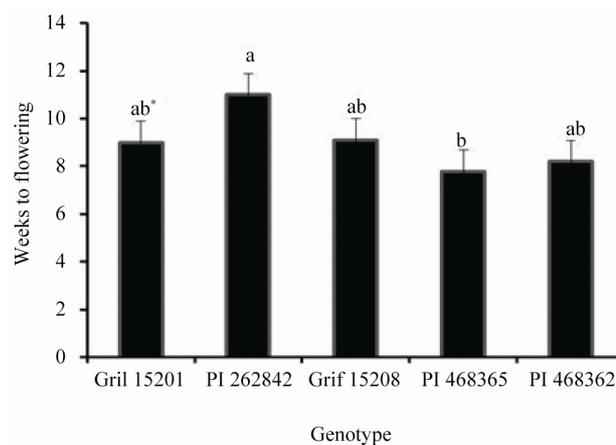


Figure 2. The time taken for *in vitro* plantlets to initiate flower buds across all day length treatments.

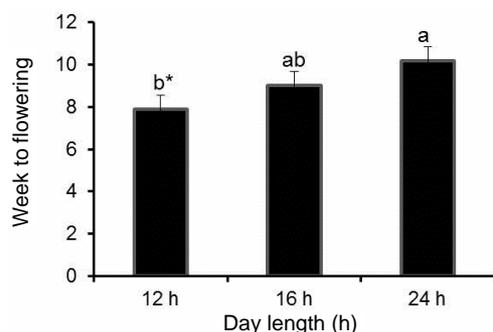


Figure 3. The time taken for *in vitro* plantlets to initiate flower buds across the five genotypes as affected by three day length treatments.

for *in vitro* plantlets to initiate flower buds across the five genotypes varied with the plantlets exposed to 12 h day-length flowering earlier than those incubated under the 24 h day-length (**Figure 3**).

Summerfield and Roberts [30] explained the difficulty involved in defining the exact day-length response of *Arachis* species since the interactions between environmental factors in many experiments were not clearly defined. Bagnall and King [31,32] later proposed that peanut should be classified as a short day plant because flowering was significantly enhanced during short-day photo periods. Thus far, many authors [33-35] continue to refer to species in the genus *Arachis* as day neutral plants with respect to their reproductive performance. The results presented in this paper agree with other field studies [31-33] indicating short day-length-induced flowering in *Arachis*.

4. Conclusion

The results from this study provide evidence of a photoperiodic reproductive response of tissue culture derived plantlets from five genotypes of *A. paraguariensis*. While *in vitro* flowering response differ among the genotypes, the highest flowering and peg formation across all the genotypes occurred under short day-length of 12 h. Hence, the observed short day-length-induced flowering should be considered while utilizing *A. paraguariensis* as a model plant for studying flowering mechanisms. Future work should focus on exposing plantlets to shorter day-lengths as well as investigating the effect of light qualities on *in vitro* flowering in *Arachis* species.

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