In vitro flowering of *Physalis angulata* L. (Solanaceae)

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RESUMO: Sementes de *Physalis angulata* germinaram *in vitro*, em meio de Murashige & Skoog (MS), apresentaram comportamento fotoblástico positivo e as plântulas assim originadas foram utilizadas para micropropagação *in vitro*, através do subcultivo de segmentos nodais de caule em meio MS . O intervalo entre subcultivos foi de 20 dias e a taxa de multiplicação média obtida ao longo de 4 subcultivos de 1:3,8 plantas. A maturidade da fonte do explante se mostrou fundamental para floração de *P. angulata in vitro*, pois a formação de brotos florais (0,6 mm) só ocorreu 3 meses após o 4º subcultivo, sendo que estes não apresentaram desenvolvimento nessas condições. Após o 5º subcultivo, a taxa de floração de ápices foi de 50% e de segmentos nodais de 45%. O desenvolvimento dos brotos florais foi mais rápido em segmentos nodais, ocorrendo a primeira ântese após 21 dias. As flores apresentaram desenvolvimento normal com pólen viável. Não foi adicionado nenhum regulador de crescimento ao longo de todo procedimento experimental.

Palavras-chave: plantas medicinais, juvenilidade, planta medicinal, Solanaceae, cultura de tecidos.

ABSTRACT: *In vitro* flowering of *Physalis angulata* L. (Solanaceae). Germination of *Physalis angulata in vitro* in Murashige & Skoog medium (MS) showed positive photoblastic characteristics. These plants were cultured using the single-node method in MS medium. The interval between subcultures was 20 days and the average multiplication rate obtained through 4 subcultures was 1: 3.8 plantlets. Flowering of *Physalis angulata* occurred 3 months after the 4th subculture, showing 0.6 mm floral buds. The mature state of the explant source was found to be fundamental for *in vitro* flowering. After 5th subculture, the rate of flowering from apexes was 50% and from nodal segments, 45%. The development of floral buds was higher and faster from nodal segments than apexes, the first anthesis occurring after 21 days. The *in vitro* flowers showed normal development and viable pollen. Plantlets transferred to soil accomplished the vegetative cycle, producing fruits after 3 months. Growing regulator was not added along the experimental procedure.

Key words: Axillary bud, juvenility, medicinal plant, Solanaceae, tissue culture.

INTRODUCTION

Physalis angulata L. is a herbaceous plant of the Solanaceae family. It is a cosmopolitan plant of the tropical zone (Braga, 1976) used in Brazil as food and in traditional medicine to treat hepatitis and rheumatism (Branch & Silva, 1983). This species showed trypanocidal activity (Freiburghaus *et al.*, 1996) and it is a source of physalin F, a steroid that has antitumor activity (Chiang *et al.*, 1992). It is normally propagated through seeds, but *in vitro* propagation offers some advantages, including the possibility of selection and clonal propagation of specific genotypes from populations and germplasm preservation, which would enable large scale production.

This work reports the *in vitro* flowering in *P. angulata* from nodal segments and shoot apexes, taking into account the influence of the time interval between subcultures. The consequences for morphogenesis of the original position of the explant on the intact plant at the time of excision was also evaluated.

MATERIAL AND METHOD

Plant Material

Plants from the State of Pará (Brazil) were the source of *Physalis angulata* L. seeds. The seeds were stored at 4° C for 10 months. The seeds were germinated and grown to the seedling stage *in vitro*. Representative specimens were selected and given to the herbarium of the botanical department of the Federal University of Rio de Janeiro under the register RFA 23907 and RFA 23908.

Germination

Seeds were washed under stirring in 5% neutral detergent for 20 minutes, followed by 3 rinses with distilled water before surface sterilization with 2% NaOCl for 4 minutes and 70% ethanol for 2 minutes, followed by 3 rinses in sterile distilled water in the laminar flow chamber. Seeds were germinated

Recebido para publicação em 25/09/02
e aceito para publicação em 25/05/03.

https://doi.org/10.70151/r64g9g47

under fluorescent white light or in the dark, in 500 ml culture vessels with 40 ml of basic MS salts (Murashige & Skoog, 1962), supplemented with 87.3 mM sucrose, 4.1 mM nicotinic acid, 1.5 mM thiamine.HCl, 0.6 mM myo-inositol, 2.4 mM pyridoxine.HCl and 0.7% agar. The pH was adjusted to 5.8 before autoclaving sterilization at 121 °C for 15 minutes.

The seeds were maintained at 25± 2 °C under 16-hour photoperiod (23.4 $mmol.m^{-2}.s^{-1}$ from a 20-W white fluorescent lamp). The criterion for germination was the rootlet protrusion and the germination rate was evaluated at 6-day intervals. Each treatment was replicated 5 times using 30 seeds per repetition.

Subcultures

Thirty days after sowing, six seedlings obtained as above described, were subcultured by single-node method every 20 days, at the same culture conditions used for germination under white light. The overall budding, rooting and multiplication rates were calculated from four successive subcultures as well as the final rate.

In vitro flowering

Development of floral buds was monitored every 10 days during the whole *in vitro* experiments. After the fourth subculture, the plants were left in the same medium for 3 or 6 months. A stereoscopic microscope was used to register the development of floral buds. Some of *in vitro plants* that developed floral meristems were subcultured 3 months after the fourth subculture using apexes and nodal segments as explants. The rate of flowering was recorded and the influence of the original position of the explant on the intact plant at the time of excision was evaluated. Five different clones were used, totaling 126 explants. The c2 statistical test was used with a 5% significance level to evaluate the difference in the flowering rates between apexes and nodal segments. Twenty days after the 5th subculture, floral buds from apexes and nodal segments were measured and their development was compared. The pollen viability was tested by staining with acetocarmine after Dickson & Bell (1974). 400 pollen grains from five *in vitro* flowers and 400 from five flowers of field plants were used. The c2 statistical test was used to a 5% significance level to evaluate the difference between *in vitro* and field pollen viability.

Acclimatization

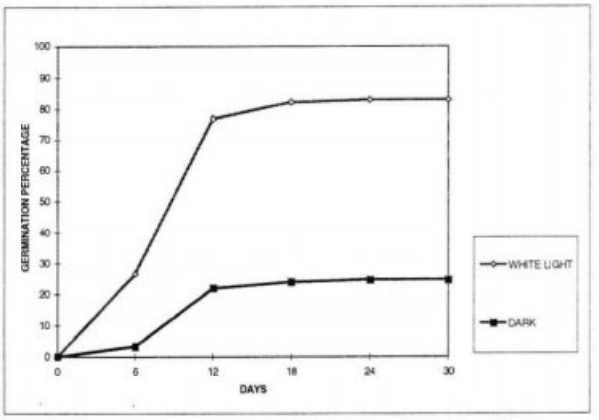
Rooted plants (100), with or without flowers, with stems measuring 5 to 7 cm were transferred individually to pots containing a mixture of sand and dark soil (1:1). These were kept in a greenhouse and after 3 weeks the survival percentage was recorded. Plants were transferred to the field, where their life cycle was observed.

RESULT AND DISCUSSION

Germination

Ten months after storage at 4 °C, seeds of *P. angulata* exhibited epigeous positively photoblastic germination, requiring 6 to 24 days to germinate on the culture medium. Germination reached 83% under white light and 25% under darkness (Figure 1). Foster & Janson (1985) stated that very small seeds, containing a little reserves usually need light to germinate, so they rapidly will produce their own nourishment through photosynthesis, soon becoming independent of the seed carbohydrates reserve.

FIGURE 1 - Effect of light on germination of *P. angulata* *in vitro* seeds



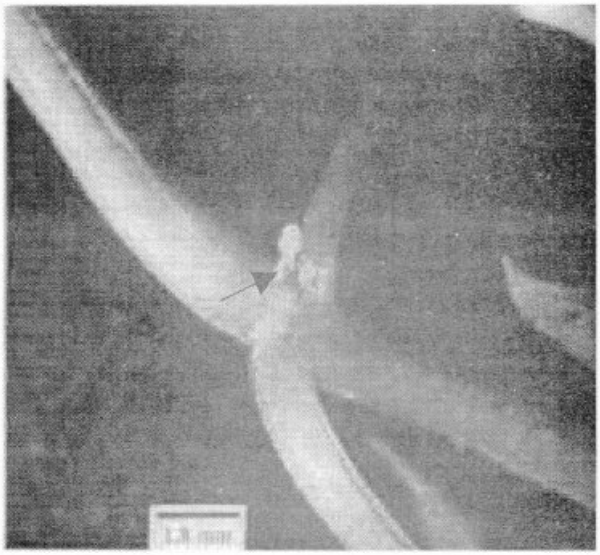
Culture of nodal segments

The addition of growth regulators to MS medium was not required for explant development. One hundred percent of the explants produced shoots and 68% rooted during the 4 subcultures. The average multiplication rate obtained through 4 subcultures was 1:3.8 plantlets, calculated taking into account the number of nodal segments per shoot produced by each explant (Table 1). No floral buds were found on any of the 181 plants observed during the four subcultures.

TABLE 1 - Rooting percentage and rate of multiplication of *P. angulata* through 4 subcultures

Subculture	Total explant	%Rooting	Multiplication rate
first	6	100	3,5
second	21	95	4,4
third	82	68	3,4
fourth	72	83	3,9

FIGURE2 - Sprouting of *P. angulata* floral bud 3 months after the fourth subculture.



Clonal propagation of this species saves space and time. Furthermore, this technique makes possible quick selection and propagation of clones with superior characteristics, as for instance, higher concentration of active principles of interest, such as physalins (Vasconcellos *et al.*,1998).

In vitro flowering

Floral buds were observed at least 3 months after the 4th subculture (Figure 2) on some plants of the 5 selected clones. These floral buds were distributed along the stem axis, however failed to develop when maintained in the same conditions for 3 additional months. The presence of floral buds in some of these plants is an evidence that the florally

FIGURE 3 - Floral buds of *P. angulata* developed 18 days after the 5th subculture.

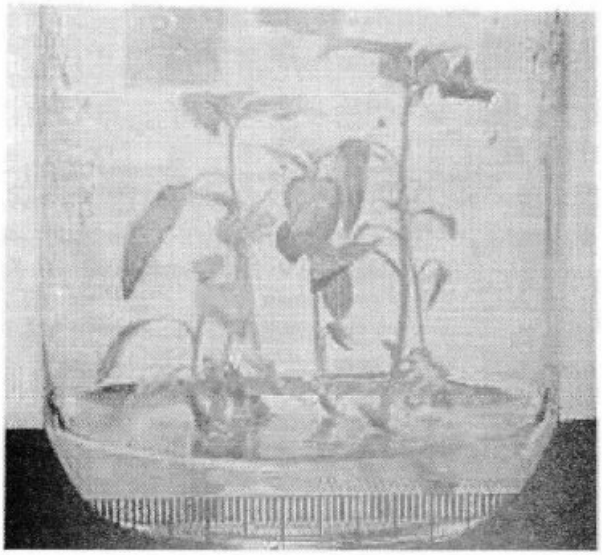
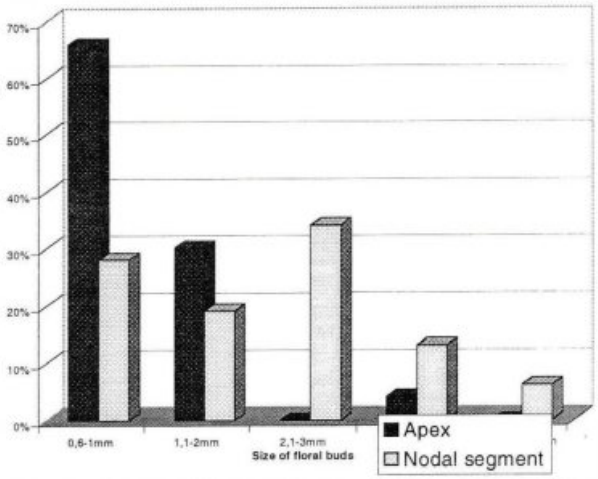


FIGURE 4 - Growth of floral buds from apexes and nodal segments of *P. angulata* 20 days after the 5th subculture.



determined state had been attained. It is important to remark that the *in vitro* flowering of *P. angulata* was obtained in Murashige & Skoog medium without growth regulators addition. However, no flowering occurred during the initial four subcultures that were performed at 20-day intervals. The maturity condition of the plant used as explant source seems to be fundamental for *in vitro* flowering of *Physalis angulata*. The period of three months required for *in vitro* flowering of *P.angulata* is very close to the 4-month vegetative cycle of *P. angulata* in the field. Juvenile plants do not flower due to an inability to produce flowering factors or to respond to them (Hackett,1995). In herbaceous plants it is classically believed that all meristems, young or old, are competent (Bernier,1988). Thus, juvenility seems not to be related to meristem incompetence in this species. The inability to develop floral buds is probably due to physiological limitations in other plant parts, especially leaves (Lang,1965).

Many authors have discussed the influence of growth regulators on *in vitro* flowering (Bernier, 1988; Scorza, 1982; Compton & Veilleux, 1992; Joshi & Nadgauda, 1997; Ochatt *et al.*, 2000; Mitrovic *et al.*, 2000; Bais *et al.*, 2000). The cytokinins exert a major influence on *in vitro* flowering (Scorza, 1982; Bais *et al.* 2000). In contrast, the fact that cytokinins are unable to induce flowering in juvenile tissues suggests that other factor(s) may be involved (Scorza & Janick, 1980). In this work growth regulators were not added to the culture medium, thus, the presence of floral buds probably are consequence of the endogenous level of hormones.

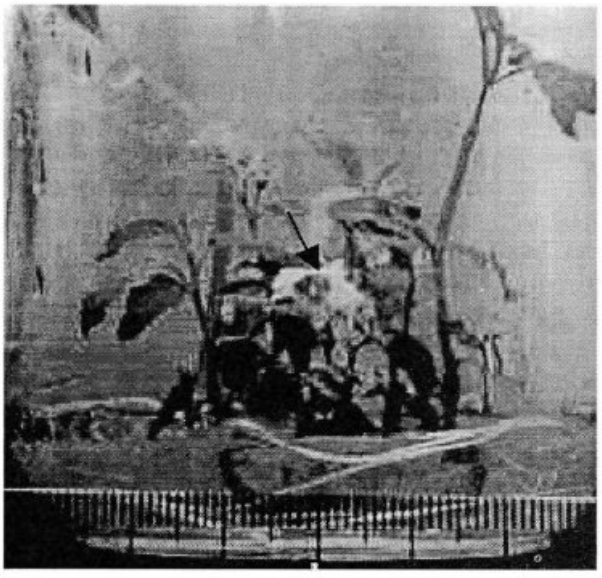
The sprouting of new floral buds and the development of the existing ones occurred following the 5th subculture of the plants (Figure 3). Studies have elucidated that the ability of *in vitro* flowering and the amount of flowers produced per explant

TABLE 2 - Flowering aspects in *P. angulata* 20 days after the 5th subculture

Clone	Explant source	Nº of explants	Nº of explants with flower	Average number of flowers per explant	Average height (cm)	Average number of new buds per explant
1	Apex	36	19	1.16	8.59	7.28
1	nodal segment	24	5	1	3.64	2.54
2	Apex	6	3	1.33	6.25	5.17
2	nodal segment	6	6	1	5.03	3.66
3	Apex	12	2	2.5	4.36	4.08
3	nodal segment	6	0	0	4.90	3.83
4	Apex	6	4	1.5	4.46	3.83
4	nodal segment	6	5	1.8	3.72	3.33
5	Apex	6	5	1.4	8.38	6
5	nodal segment	18	11	1.09	6.21	3.28
Total	Apex	66	33 a	1.33 b	7.21 c	6.08 e
Total	nodal segment	60	27 a	1.18 b	4.68 d	3.08 f
Total	General	126	60	1.27	6.01	4.65

decreases as the distance from the apex increases (Scorza, 1982; Scorza& Janick, 1980; Jumin & Nito, 1996). In this work, differences in flowering and the amount of flowers per explant among apexes and nodal segments was not statistically significant (Table 2). According to Compton & Veilleux (1992), the position of the explant on the intact plant is extremely important when attempting to obtain de novo flowers on thin cell layer explants of photoperiodically sensitive tobacco cultivars. In contrast, the independence of de novo flowering in *P. angulata* in relation to the original position of the explant on the main stem, suggests that the combination of promoters and inhibitors along the stem at the time of excision is potentially alike and demonstrates that the explants near the base are also able to flower.

FIGURE 5 - Anthesis of *P. angulata* normal flower, 21 days after the 5th subculture.



The greater number of newly formed vegetative buds from apexes strongly indicates the use of this explant may optimize the multiplication rates on micropropagation process. Taller plants originated from apexes in relation to those originated from nodal segments demonstrate the time lapse required to break the dormancy of axilary buds, thus promoting explant vegetative growth. The explants originated from apexes present immediate growth since stem apical meristem is in continuous division.

The growth of floral buds was greater from nodal segments than from apexes. Taking into account that in the apexes the endogenous concentration of auxin is greater, it is possible suppose that this hormone could interfere in floral determination. On the other hand, nodal segments had their main sources of auxin removed (apical meristem and young leaves), changing the endogenous hormonal level, affecting the development of the plant elongation, thus making the plant direct resources mainly to the development of floral buds.

First anthesis happened on plants originated from nodal segments 21 days after the 5th subculture (Figure 5). The flowers presented normal development and 60% of the pollen from *in vitro* flowers was viable. Other authors had presented results showing the *in vitro* flowers as able to produce viable pollen (Joshi & Nadgauda, 1997; Zhang & Leung, 2000). During a preliminary experiment, some plants were hyperhydric, their flowers showing abnormal development with atrophied or absent petals, stamens, carpels and only the calix was observed. According to Scorza (1982) typical abnormal development of flowers produced *in*

vitro has provided new, yet fragmented, insights into the control of floral morphogenesis and the growth regulators have been implicated as major controlling factors. In this work no growth regulator was added throughout the whole experimental procedure so that malformed flowers arose probably due to other factors, the physical state of culture media and high humidity in the culture flasks not being disregarded.

Acclimatization

Eighty five percent of the plants transferred to soil survived and all of them exhibited normal phenotypes. Flowering and fruiting occurred normally, after 3 months in the field, same time needed to *in vitro* plants produce flowers. Pandey (1957) relates the occurrence of gametophytic self-incompatibility in plants from the *Physalis* genus. In our study, the plants from all 5 clones were found to be self-compatible, generating fruits with viable seeds from self-pollination. The pollen viability of plants from the field was 83%, demonstrating that *in vitro* conditions influence this factor. The field grown plants will be used as explant sources for further experiments.

The simplicity of the *in vitro* culture presented in this work in addition to the short reproductive cycle, make *Physalis angulata* an excellent model for studies of propagation, flowering and for comparing the development patterns of *in vitro* plants and those cultured in the field.

ACKNOWLEDGMENT

Financial support: CNPq, CAPES, Fundação Universitária José Bonifácio.

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