

ORGANOGENESIS FROM INTERNODAL SEGMENTS OF YELLOW PASSION FRUIT

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ABSTRACT: Organogenesis induction from internodal segments of yellow passionfruit (*Passiflora edulis* f. *flavicarpa*) was obtained in a medium supplemented with 1.0-4.0 mg L⁻¹ benzylaminopurine (BAP). Callus proliferation and adventitious shoot development were analyzed by light and scanning electron microscopy. Callus proliferation and adventitious shoots were observed simultaneously and continuously, with asynchronous development of shoots in the explants. Proliferation of callus was observed in the explant cut ends in all treatments, including the control. The results show that concentrations of 1.0 or 2.0 mg L⁻¹ BAP gave better rate of organogenesis. Higher concentrations were efficient for the induction of adventitious buds and, were, however, detrimental for subsequent shoot development. Growth and rooting of shoots were obtained in half strength medium without growth regulators.

Key words: *Passiflora edulis* f. *flavicarpa*, adventitious shoots, light microscopy, scanning electron microscopy, benzylaminopurine

ORGANOGENESE A PARTIR DE SEGMENTO INTERNODAL DE MARACUJÁ AMARELO

RESUMO: Estudou-se a indução de organogênese em segmentos internodais de maracujá amarelo (*Passiflora edulis* f. *flavicarpa*) cultivados em meio contendo 1,0 a 4,0 mg L⁻¹ de benzilaminopurina (BAP). A formação de calo e o desenvolvimento de gemas adventícias foram acompanhados através de microscopia ótica e eletrônica de varredura, que mostrou sua ocorrência simultânea e contínua, com observação de gemas em diversos estádios de desenvolvimento em um mesmo explante. Houve proliferação de calos nas extremidades dos explantes em todos os tratamentos, inclusive no controle. A avaliação dos efeitos do BAP na indução de gemas adventícias mostrou que as concentrações de 1 ou 2 mg L⁻¹ foram mais adequadas. Doses superiores a 2 mg L⁻¹, apesar de eficientes para indução, foram prejudiciais ao crescimento das gemas. Crescimento e enraizamento das gemas adventícias foram obtidos em meio de cultura MS sem reguladores de crescimento e com a concentração de sais reduzida pela metade.

Palavras-chave: *Passiflora edulis* f. *flavicarpa*, gemas adventícias, microscopia óptica, microscopia eletrônica de varredura, benzilaminopurina

INTRODUCTION

Passiflora edulis f. *flavicarpa*, yellow passionfruit, is a tropical fruit, native to Brazil and widely distributed throughout the tropics and subtropics. From its fruits, an acidic juice is obtained and used for juices, sherbets, jams and jellies (Purseglove, 1968). Leaves of this species contain alkaloids with potential use for the pharmaceutical industry (Lotschert & Beese, 1989).

It is a perennial plant that can be propagated by seeds, cuttings, air-layering, or grafting. Commercial areas, however, are based on seedlings, which generate high levels of genetic variability (Drew, 1997). *In vitro* culture can be useful for clonal propagation of superior genotypes and disease resistant rootstocks, and as a tool for genetic transformation.

Although this species is resistant to *Fusarium* wilt (Gardner, 1989) and nematodes (Drew, 1997), other fungi, viruses and bacteria, are responsible for significant losses in yellow passionfruit production. The use of meristem

culture for the production of virus-free plants has been reported with partial success (Chiari et al., 1994), however, there is always the risk of reinfection of perennial plants. The use of genetic transformation is probably the most indicated technique for production of disease resistant plants (Drew, 1997).

In vitro culture studies using different explants of *Passiflora edulis* f. *flavicarpa*, under different culture conditions, have been reported (Moran Robles, 1978, 1979; Drew, 1991; D'Utra Vaz et al., 1993; Carvalho & Segura, 1994; Dornelas & Vieira, 1994). *In vitro* studies of this and other *Passiflora* species, have been reviewed by Drew (1997).

Description and optimization of tissue culture protocols is important for the definition of strategies focusing large-scale multiplication and genetic transformation. Hence, this research aimed to characterize the induction and culture conditions for obtaining adventitious organogenesis from internodal segments of *Passiflora edulis* f. *flavicarpa*. Morphological and histological observations of the process are presented.

MATERIAL AND METHODS

Tissue Culture: Seeds of yellow passionfruit were germinated in the greenhouse. Stems were obtained from approximately two-month-old seedlings, washed in tap water and treated with an antibiotic solution (1.0 g L⁻¹ Agrimicine) for one hour to avoid bacterial contamination, according to preliminary tests. The stems were then disinfested in a solution of commercial bleach (0.7% chlorine) and Tween 20 (0.1%) for 20 minutes. After four rinses in sterile water, the stem segments were sectioned to obtain one-centimeter-long internodal segments between the first and third nodes.

The explants were placed horizontally in petri dishes with MS medium (Murashige & Skoog, 1962) supplemented with 6.5 g L⁻¹ agar and different BAP concentrations (0, 1, 2, 3 and 4 mg L⁻¹). The pH of the medium was adjusted to 5.8 prior to autoclaving (20 minutes at 120 C and 1 atm). Cultures were maintained at 27 ± 3 C under a 16-hour photoperiod. A completely randomized design with five replications was used, with each plot consisting of two Petri dishes with eight explants in each.

Cultures were evaluated eighteen days after inoculation, and the number of explants with callus and adventitious buds was recorded. The responsive explants were transferred to MS medium without growth regulators, with half of the explants being cut transversally, resulting in another experiment (factorial 2x4) where it was evaluated the effect of the cut on the growth of adventitious buds. This second experiment was scored after twenty days in culture, by recording the number of buds with initial leaf expansion per explant.

Several subcultures followed at 3-week intervals. Adventitious buds with approximate 0.5 cm were subcultured to half strength MS medium for better elongation and rooting. Rooted plantlets were acclimatized under mist in the greenhouse.

Light Microscopy (LM): Samples were fixed in a solution of freshly prepared paraformaldehyde (3% w/v) and glutaraldehyde (2% v/v) in 0.2 M cacodylate buffer (overnight, at 4C, pH 7.2). Low vacuum was applied during the first hour of fixation. Dehydration was done at room temperature in a series of ethanol, followed by infiltration and embedding in Historesin (hydroxyethylmethacrilate). Polymerization was completed after 36 to 48 hours at room temperature. Sections 6 to 8-mm-thick were cut with a tungsten carbide knife on a Leica rotary microtome, floated on water on glass slides, and dried at room temperature. Sections were stained with toluidine blue.

Scanning Electron Microscopy (SEM): Samples were fixed in paraformaldehyde (2% w/v) and glutaraldehyde (2% v/v) in 0.05 M cacodylate buffer and calcium chloride (0.001 M) at pH 7.2 for 1 hour, followed by three rinses with 0.05 M sodium cacodylate (10 minutes

each). The samples were post-fixed in osmium tetroxide (1%) in sodium cacodylate (0.1 M) for one hour. After three rinses in distilled water the samples were dehydrated through a graded acetone series and critical point dried through liquid carbon dioxide. The samples were then mounted on stubs, sputter coated with gold, examined and photographed on a Zeiss microscope (DSM 940A) at 5-10 kV.

RESULTS AND DISCUSSION

In the days following explant inoculation, a general swelling was observed in explants of all treatments, followed by cell proliferation on the cut ends. By the second week, leaf primordia interspersed with callus could be observed through scanning electron microscopy (Figures 1a, b).

Callus proliferation was observed in most of the explants in all treatments, including the control, mainly on the surface of the cut ends. The regression curve shows a maximum percentage of explants with callus at 1.3 mg L⁻¹ BAP (Figure 2). Most of the explants had callus forming in both cut ends (TABLE 1). Although this callus tissue did not appear to be organogenic, with large, loose cells, adventitious buds were always observed interspersed with callus cells (Figures 1c, d, f).

Bud proliferation occurred associated with callus formation in a continuous pattern. Buds in different developmental stages were observed in the same area of explants. Initially leaf primordia with a conical shape were observed (Figure 1a), following the development of the lateral meristems of the leaves (Figure 1b). In developing leaves, stomata were already observed, always completely opened due to the high humidity characteristic of *in vitro* culture (Figures 1b, e).

Exposure of internodal segments to medium with different concentrations of BAP was efficient for the formation of adventitious buds (Figure 3). The highest percent of explants forming adventitious buds was observed with 1.0 or 2.0 mg L⁻¹ BAP. According to the regression equation, the best results would be obtained with 2.1 mg L⁻¹ BAP, with the percent of responsive explants decreasing with further increase in BAP concentration. A small percentage of the explants formed adventitious buds in the control, only in the apical part, while in the explants treated with BAP adventitious buds were observed in both basal and apical ends of the explant

TABLE 1 - Percentage of distribution of the explants forming callus, according to its occurrence in the basal and/or apical end of internodal segments of yellow passionfruit.

| | BAP (mg L ⁻¹) | | | | |
|----------------|---------------------------|------|-----|------|------|
| | 0 | 1.0 | 2.0 | 3.0 | 4.0 |
| Apical only | 0 | 0 | 0 | 3.9 | 6.3 |
| Basal + Apical | 79.2 | 98.7 | 100 | 86 | 85.6 |
| Basal only | 20.8 | 1.2 | 0 | 10.1 | 8.1 |

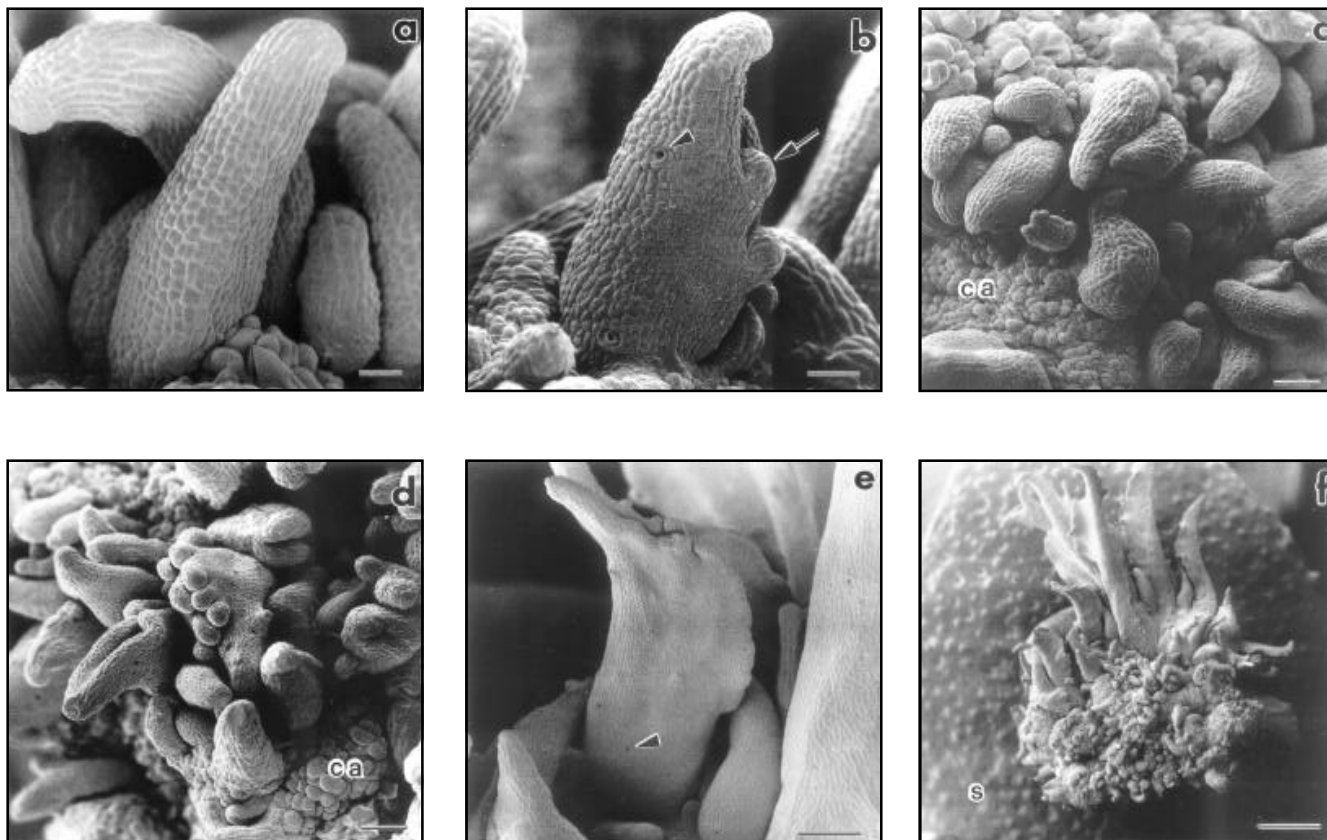


Figure 1 - Scanning electron micrographs of organogenesis from internodal segments of yellow passionfruit induced in medium containing 2 mg L⁻¹ BAP: a. initial stage of development of leaf primordia (bar = 50 μm); b. leaf primordium with development of lateral meristems (arrow) and stomata already differentiated (point) (bar = 50 μm); c, d. several buds/leaf primordia interspersed with callus cells (ca) (bar = 100 μm); e. detail of a developing leaf with differentiated stomata (point), (bar = 200 μm); f. transversal surface of an internodal segment showing the development of buds and leaf primordia at different stages (bar = 1 mm, s = stub).

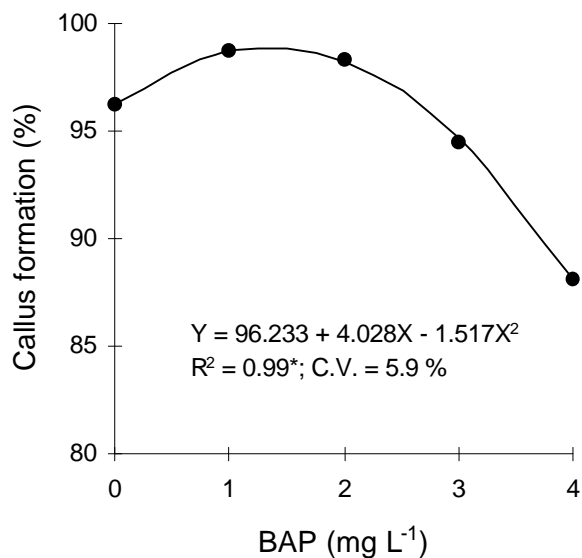


Figure 2 - Percent of internodal segments of yellow passionfruit forming callus after 18 days in medium containing different BAP concentrations (dots indicate observed data, and the line represents the data estimated by the regression equation Y, R² = coefficient of determination, C.V. = coefficient of variation).

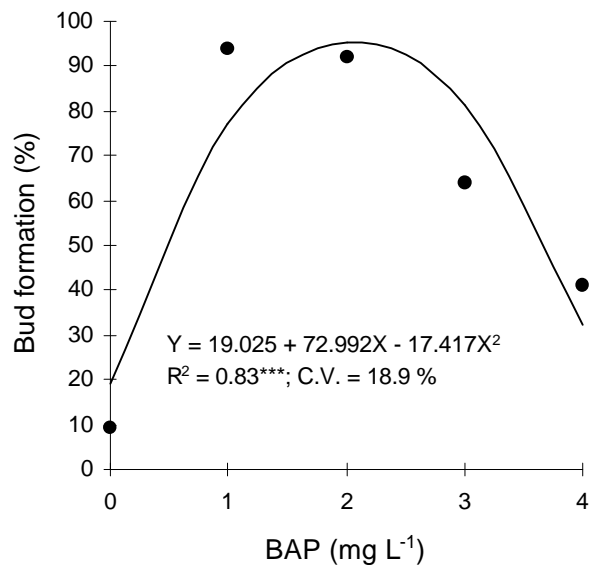


Figure 3 - Percent of internodal segments of yellow passionfruit forming adventitious buds after 18 days in medium containing different BAP concentrations (dots indicate observed data, and the line represents the data estimated by the regression equation Y, R² = coefficient of determination, C.V. = coefficient of variation).

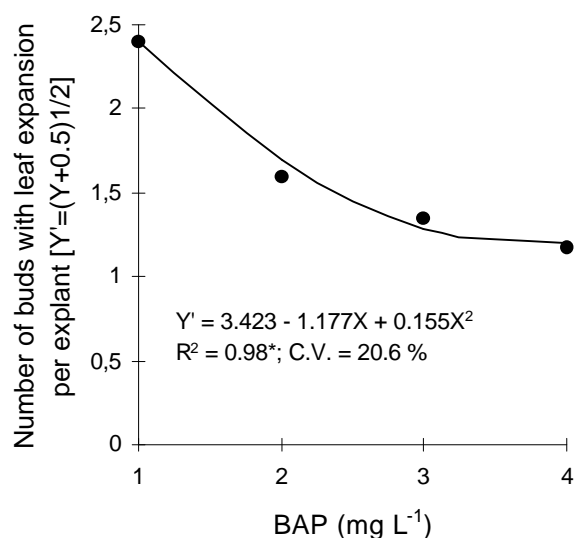


Figure 4 - Effect of the use of different concentrations of BAP for induction of adventitious buds in internodal segments of yellow passionfruit on the number of buds with leaf expansion (dots indicate observed data, and the line represents the data estimated by the regression equation Y' , R^2 = coefficient of determination, C.V. = coefficient of variation).

TABLE 2 - Percentage of distribution of explants forming adventitious buds, according to its occurrence in the basal and/ or apical end of internodal segments of yellow passionfruit.

| | BAP (mg L ⁻¹) | | | | |
|----------------|---------------------------|------|------|------|------|
| | 0 | 1.0 | 2.0 | 3.0 | 4.0 |
| Apical only | 100 | 56.4 | 25.1 | 88.1 | 68.7 |
| Basal + Apical | 0 | 43.6 | 74.9 | 11.9 | 31.2 |
| Basal only | 0 | 0 | 0 | 0 | 0 |

(TABLE 2). Treatments with 1.0 or 2.0 mg L⁻¹ BAP showed a higher percent of explants forming buds in both ends. This can be advantageous for co-cultivation with *Agrobacterium*, since there is a larger area of contact. However, it is necessary to verify if this behavior is maintained after bacterium infection.

The transversal cut made to separate and transfer the area of the explant where the adventitious buds were formed did not influence subsequent growth of those buds. A larger number of buds showing leaf expansion was observed from explants that had been initiated in medium containing 1.0 mg L⁻¹ BAP, with a decrease when higher concentrations were used for induction. Dornelas and Vieira (1994) also report the disadvantageous effect of 2.0 mg L⁻¹ BAP in the induction medium on further bud elongation in different *Passiflora* species. This effect could have been caused by the residual effect of BAP that can promote larger numbers of buds per explant in detriment to bud elongation.

The use of half-strength MS was effective for rooting of the individualized buds. Similar results were

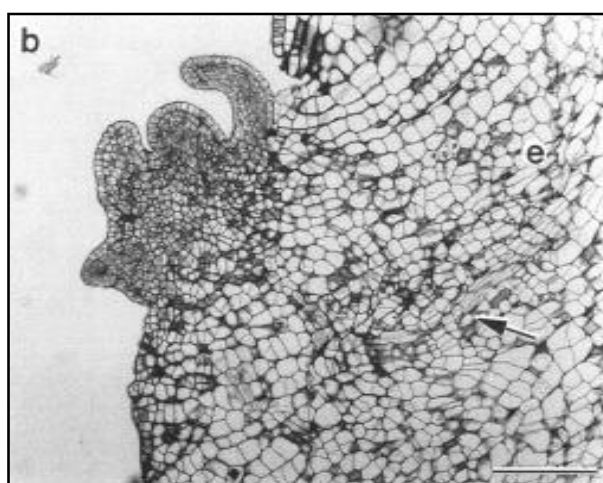
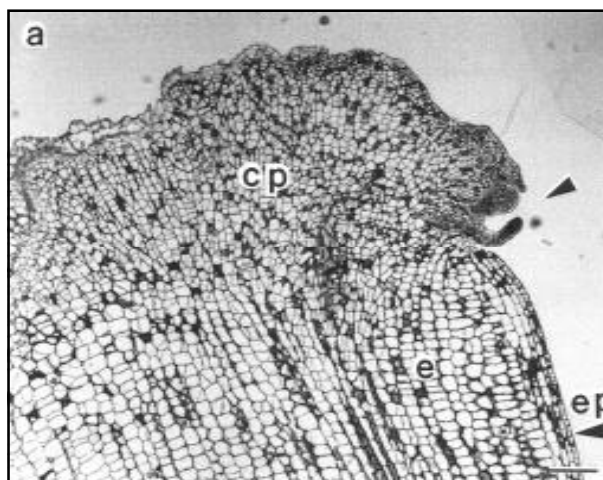


Figure 5 - Longitudinal sections showing adventitious bud initiation from internodal segments of yellow passionfruit, after 20 days in medium with BAP. a. cut region of the explant (e) showing the proliferation of cells (cp) and formation of a shoot bud (point); b. a closer view of a shoot bud and region of elongating cells showing initial differentiation of procambium (arrow) (bars = 200 μ m). ep = explant epidermis.

reported by Dornelas & Vieira (1993, 1994). Moran Robles (1978), Kantharajah & Dodd (1990) and Dornelas & Vieira (1994) previously reported the absence of growth regulators for rooting of *Passiflora* species in vitro.

Longitudinal sections at day 20 showed an extensive proliferation of cells on the cut region of the explants. Adventitious bud formation directly from this region was observed, as well as differentiation of a procambial tissue (Figure 5). A superficial initiation of adventitious buds, as observed in this work, is favorable for *Agrobacterium* infection. Studies are underway to determine the efficiency of this type of explant for co-cultivation with *Agrobacterium* for future work on genetic transformation aiming for the development of more tolerant or resistant clones to diseases that affect this tropical species.

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