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IN VITRO CONTROL OF ADVENTITIOUS BUD DIFFERENTIATION BY INORGANIC MEDIUM COMPONENTS AND SILVER THIOSULFATE IN EXPLANTS OF *PASSIFLORA EDULIS* F. *FLAVICARPA*

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SUMMARY

Hypocotyl and leaf explants from *Passiflora edulis* F. *flavicarpa* were evaluated for morphogenesis when cultured on several nutrient media supplemented with benzyladenine and indoleacetic acid. The effect of silver thiosulfate on growth-regulator-induced morphogenesis was also investigated. Murashige and Skoog medium was more effective than woody plant medium in promoting adventitious bud differentiation. The omission of ammonium or nitrate from the Murashige and Skoog medium and a disequilibrium from the Murashige and Skoog nitrate:ammonium ratio drastically reduced the bud-forming capacity of the explants. The inclusion of silver thiosulfate in the culture medium significantly increased the differentiation and development of adventitious shoots. Regenerated shoots were excised and induced to root on basal Murashige and Skoog medium. Plants were transplanted to pots and grown *ex vitro*.

Key words: Passiflora edulis F. *flavicarpa*; adventitious buds; ammonium and nitrate ratios; ethylene.

INTRODUCTION

Passiflora edulis F. *flavicarpa* Deg. (yellow passionfruit), a woody perennial climber plant, is one of the most economically important species of the family Passifloraceae in tropical regions of South America. The yellow passionfruit is mainly grown for its edible fruits and, to a lesser extent, for ornamental and pharmaceutical purposes (Maluf et al., 1991; Vanderplank, 1991). Although clonal propagation from juvenile tissues of Passifloraceae has been described previously (*see* Dornelas and Vieira, 1994, and references therein; Kawata et al., 1995), these studies focused mainly on the effects of growth regulators on adventitious bud differentiation. In order to obtain maximum efficiency in plant regeneration, a quantitative approach leading to the optimization of the inorganic media components is also required.

Plant cells and tissues produce ethylene when cultured *in vitro* (George, 1993). The accumulation of this gaseous hormone is generally viewed as a noxious phenomenon that should be avoided in the *in vitro* environment (Krikorian, 1995). Several chemicals and environmental factors are known to inhibit ethylene action (Reid, 1995), although their effects on *in vitro* morphogenesis show a wide range of variability depending on species and tissues in culture (Biddington, 1992). *Passiflora* spp. show high rates of ethylene production (Ludford, 1995), which might limit the morphogenic potential of the cultured explants. To date, however, no studies have been reported that relate morphogenesis to ethylene inhibition in *Passiflora* cultures.

The objective of this research was to study the effects of inorganic medium components on caulogenesis from cultured hypocotyl and leaf explants of *P. edulis* F. *flavicarpa*. The effect of the addition of silver thiosulfate (STS), a stable, mobile, and nonphytotoxic inhibitor of ethylene action shown to have positive effects on *in vitro* culture of several species (Biddington, 1992), was also tested.

MATERIALS AND METHODS

Plant Material

Hypocotyl sections (0.5 cm long) and leaf explants (0.5 cm^2) of the last four fully expanded leaves, isolated from 40-d-old seedlings of *Passiflora edulis* F. *flavicarpa* were used in the experiments. Seedlings were grown from seeds germinated under sterile conditions. Seeds harvested from field-grown plants (University of Rio Grande do Sur, Brazil) were imbibed for 1 h in sterile distilled water at 45° C, surface-sterilized with 2% chloramine T (Merck, Darmstadt, Germany) and 0.1% Tween-20 (Panreac) for 30 min, rinsed with sterile distilled water, and germinated aseptically in petri dishes on solid medium (2% sucrose and 0.8% Difco-Bacto agar in distilled water, pH 5.7). Seedlings were transferred to glass culture tubes containing MS salts and vitamins (Murashige and Skoog, 1962), 2% sucrose, and 0.8% Difco-Bacto agar (pH 5.7).

Culture Procedures

The basal media used in the experiments were based on MS (Murashige and Skoog, 1962) or woody plant medium (WPM) (Lloyd and McCown, 1980) formulations. The media included MS or WPM macronutrients, micronutrients and vitamins of Murashige and Skoog (1962), 3% sucrose, and 0.8% Difco-Bacto agar (pH 5.7). Growth regulators were added to the media before autoclaving (20 min at 120° C, 1×10^5 Pa). Vessels used in the experiments were 15- × 100-mm petri dishes or 150- × 25-mm glass tubes with 30 ml each of nutrient medium for agar-solidified cultures, and 125 Erlenmeyer flasks with 30 ml of liquid medium for continuously agitated (50 rpm) cultures. Cultures were maintained in a growth chamber at 26 ± 2° C and a 16 h photoperiod with light supplied by Sylvania (GTE Gro-lux, F36W/GRO, Erlangen, Germany) fluorescent tubes (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance at culture level).

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The following experimental modifications in the culture conditions were assayed:

(a) *Nutrient medium and changes in the concentration and source of* NH_4^+ *and* NO_3^-. Hypocotyl and leaf (abaxial surface to the medium) explants were cultured in media supplemented with 5 μ*M* benzyladenine (BA) and 2 μ*M* indoleacetic acid (IAA).

In the first experiment, explants were cultured on MS medium, WPM medium, or modified MS or WPM media. The particular modifications tested were: (1) modified WPM (MWPM): both NH_4^+ and NO_3^- levels were raised to those of MS medium by adding 1900 mg/l KNO_3 and 1650 mg/l NH_4NO_3; (2) omission of NO_3^- or NH_4^+ from MS and WPM media, but the total nitrogen level was maintained constant by adding 4485 mg/l $NH_4H_2PO_4$ (MS without NO_3^-); 964 mg/l $(NH_4)_2SO_4$ (WPM without NO_3^-), 4160 mg/l KNO_3 (MS without NH_4^+) or 556 mg/l $Ca(NO_3)_2 \cdot 4H_2O$ and 505 mg/l KNO_3 (WPM without NH_4^+).

In the second experiment, nitrate to ammonium ratio from MS medium was modified using the following NO_3^-:NH_4^+ concentrations (m*M*): 3.5:56.5; 12.0:48.0; 30.0:30.0; 40.0:20.0; 48.0:12.0; and 56.5:3.5. Inorganic nitrogen sources were KNO_3 and NH_4Cl. A control treatment including original MS salt formulation (NO_3^-:NH_4^+, 40:20) was also tested.

(b) *Effect of silver thiosulfate.* Explants were initially maintained for 16 h in liquid MS medium (with or without 8 m*M* STS) supplemented with 50 μ*M* IAA and BA. Subsequently, explants were transferred to agar-solidified MS medium with or without growth regulators (8.8 μ*M* BA and 2.7 μ*M* IAA) and/or 8 μ*M* STS.

In all experiments, each treatment contained 12 replications (12 dishes with 5 explants each) and culture time was 45 d. Experiments were conducted at least twice. Cultures were examined for percent of explants with elongating buds and number of adventitious shoots per initial explant.

Root Induction

Rooting experiments were carried out with shoots longer than 1 cm excised from proliferating cultures. A minimum of 16 shoots per initial treatment were excised and transferred individually to tubes containing basal MS medium. Rooting percentage and the number of roots per shoot were evaluated after 30 d.

Plantlets were transplanted individually to 100-ml pots containing a medium of 2:1 peat moss:perlite and maintained for 20–30 d in a growth chamber at 75% relative humidity, 25 ± 2° C and a photoperiod of 18 h (90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance at plant level). Before transplanting, all the leaves were removed and roots pruned to 3 cm from the base. Subsequently, plants were grown in a greenhouse (2–3 mo.) and then transferred to field conditions.

Statistical Analysis

Significance of treatment effects were determined using analysis of variance (Statgraphics program, version 6, Manugistics, Rockville, MD). Caulogenic percentage data were subjected to arcsin transformation before analysis. Variations among treatment means were analyzed using Tukey's (1953) procedure. Data sets were combined before analysis of variance.

Results and Discussion

Adventitious bud primordia formed directly from hypocotyl and leaf explants cultured in the presence of BA and IAA. These results are somewhat different from that of Moran Robles (1978, 1979), Kantharajah and Dodd (1990), and Dornelas and Vieira (1994), who reported that direct organogenesis from several explants of *Passiflora* spp. occurred on media supplemented with BA only. The number of buds per explant was difficult to quantify because they usually appeared in compact clusters, especially from the leaf explants; therefore, only those elongating buds identified by the naked eye were recorded.

Effect of Salt Formulation on Bud Induction

MS medium was more effective than WPM medium in promoting adventitious bud differentiation from cultured hypocotyls or leaves of *P. edulis* F. *flavicarpa* (Table 1). The addition of NH_4NO_3 and KNO_3 to MWPM, which equals the concentration of ammonium and nitrate in medium MS, did not improve the bud-forming capacity of the hypocotyls, but favored significantly this response from leaf explants (Table 1). WPM contains a lower concentration of both ammonium (5 m*M*) and nitrate (9.8 m*M*) than MS medium (20.6 and 39.4 m*M*, respectively). Thus, the superiority of the latter might be attributed to the higher total nitrogen content of this medium. Furthermore, the omission of NO_3^- or NH_4^+ from MS medium or WPM, as well as slight deviations from the original nitrate to ammonium ratio of MS medium, drastically reduced or nullified the bud-forming capacity of the explants (Tables 1 and 2). Nitrogen constitutes one of the major nutrients required by cultured explants for growth and development. The level and form in which this macronutrient is supplied to the cultures influences not only growth rate and metabolic activity of the plantlets, but also interferes with cell morphology and tissue development (Thorpe et al., 1989).

TABLE 1

EFFECT OF SEVERAL NUTRIENT MEDIA ON ADVENTITIOUS BUD DIFFERENTIATION FROM HYPOCOTYL AND LEAF EXPLANTS OF *PASSIFLORA EDULIS* F. *FLAVICARPA*. EXPLANTS WERE CULTURED IN THE PRESENCE OF 5 μ*M* BENZYLADENINE (BA) AND 2 μ*M* INDOLEACETIC ACID (IAA)

Nutrient medium	Caulogenic Explants (%)[a]		Number of Shoots/Explant[a]	
	Hypocotyls	Leaves	Hypocotyls	Leaves
MS	100a	50 a	3.8 a	0.7 a
WPM	92 a	0 b	2.2 b	0.0 b
MWMP*	92 a	17 b	1.8 b	0.6 a
MS without NO_3^-	0 c	0 b	0.0 c	0 b
MS without NH_4^+	33 b	0 b	0.5 c	0 b
WPM without NO_3^-	0 c	0 b	0.0 c	0 b
WPM without NH_4^+	8 bc	0 b	0.2 c	0 b

*Modified WPM medium (with NH_4^+ and NO_3^- levels raised to those of MS medium). MS = Murashige and Skoog medium; WPM = woody plant medium; MWPM = modified woody plant medium.

[a]For each column, letters followed by the same letter are not significantly different according to the Tukey's test at $P = 0.05$, n = 12.

Effect of Silver Thiosulfate on Bud Induction

A 16-h exposure of the cultures to 50 μ*M* BA- and IAA-supplemented liquid medium, with or without 8 μ*M* STS, did not produce buds when hypocotyl or leaf explants were transferred to agar-solidified MS medium without growth regulators (data not shown). Adventitious bud differentiation required the transfer of the pretreated explants to a medium supplemented with growth regulators. STS did not affect the percentage of hypocotyls producing buds. In contrast, this compound significantly increased the frequency of buds from leaf explants when added to a medium supplemented with 8.8 μ*M* BA and 2.7 μ*M* IAA (Table 3). Furthermore, STS also delayed senescence of the primary explants, especially the leaf explants. A comparison of data in Tables 1 and 3 shows a differential response in the bud-forming capacity of the leaf explants. This seems to be related to the differences in plant growth regulators added to the media.

The mean number of shoots formed per hypocotyl or leaf explant was significantly affected by the presence of STS in either liquid

TABLE 2

EFFECT OF NO_3^-: NH_4^+ RATIO ON ADVENTITIOUS BUD DIFFERENTIATION FROM HYPOCOTYL AND LEAF EXPLANTS OF *PASSIFLORA EDULIS* F. *FLAVICARPA*. EXPLANTS WERE CULTURED IN THE PRESENCE OF 5 μ*M* BENZYLADEINE (BA) AND 2 μ*M* INDOLEACETIC ACID (IAA)

			Caulogenic Explants (%)[a]		Number of Shoots/Explant[a]	
NO_3^-:NH_4^+	NO_3^- (mM)	NH_4^+ (mM)	Hypocotyls	Leaves	Hypocotyls	Leaves
1:16	3.5	56.5	0 c	0 a	0.0 e	0.0 a
1:4	12.0	48.0	100 a	4 a	1.6 b	0.1 a
1:1	30.0	30.0	95 a	4 a	1.5 bc	0.1 a
2:1*	40.0	20.0	100 a	12 a	2.4 a	0.2 a
2:1	40.0	20.0	100 a	12 a	1.9 b	0.2 a
4:1	48.0	12.0	85 a	4 a	1.1 c	0.1 a
16:1	56.5	3.5	45 b	0 a	0.6 d	0.0 a

*Control Murashige and Skoog (MS) medium (20 m*M* KNO_3 and 20 m*M* NH_4NO_3)

[a]For each column, letters followed by the same letter are not significantly different according to the Tukey's test at $P = 0.05$, n = 12.

TABLE 3

EFFECT OF SILVER THIOSULFATE (STS) ON ADVENTITIOUS BUD DIFFERENTIATION (PERCENT OF CAULOGENIC EXPLANTS) FROM HYPOCOTYL AND LEAF EXPLANTS OF *PASSIFLORA EDULIS* F. *FLAVICARPA*. EXPLANTS WERE CULTURED ON MURASHIGE AND SKOOG (MS) MEDIUM SUPPLEMENTED WITH 8.8 μ*M* BENZYLADENINE (BA) AND 2.7 μM INDOLEACETIC ACID (IAA)

	Hypocotyls			Leaves		
	STS in Culture Medium (μ*M*)			STS in Culture Medium (μ*M*)		
STS Pulse[a] (μ*M*)	0	8		0	8	Mean[b]
0	100	100		4	42	23 a
8	100	100		12	21	17 a
			Mean[b]	8 b	31 a	

[a]16 h in liquid MS medium supplemented with 50 μ*M* BA and IAA.

[b]Values followed by the same letter are not significantly different according to the Tukey's test at $P = 0.05$, n = 12.

TABLE 4

EFFECT OF SILVER THIOSULFATE (STS) ON ADVENTITIOUS BUD DIFFERENTIATION (NUMBER OF SHOOTS/CULTURED EXPLANT) FROM HYPOCOTYL AND LEAF EXPLANTS OF *PASSIFLORA EDULIS* F. *FLAVICARPA*. EXPLANTS WERE CULTURED ON MURASHIGE AND SKOOG (MS) MEDIUM SUPPLEMENTED WITH 8.8 μ*M* BENZYLADENINE (BA) AND 2.7 μ*M* INDOLEACETIC ACID (IAA)

	Hypocotyls[b]		Leaves[b]	
	STS in Cuture Medium (μ*M*)		STS in Culture Medium (μ*M*)	
STS Pulse (μ*M*)[a]	0	8	0	8
0	3.3 b	7.4 a	0.1 b	2.6 a
8	5.1 b	3.9 b	0.5 b	0.6 b

[a]16 h in liquid MS medium supplemented with 50 μ*M* BA and IAA.

[b]For each explant, values followed by the same letter are not significantly different according to the Tukey's test at $P = 0.05$, n = 12.

induction medium or agar-solidified culture medium. A significant interaction between these two factors was also evident. Thus, the highest number of shoots per explant was obtained when STS was incorporated to the agar-solidified culture medium (Table 4).

Because silver ions are potent inhibitors of ethylene action (Beyer, 1976), our results suggest that the morphogenic capacity of *P. edulis* F. *flavicarpa* was limited by the ethylene accumulated in the culture vessels or cultured material. The effects of ethylene on explant growth and morphogenesis seem to be species-specific. This hormone may promote organogenesis or embryogenesis in some plant species (Kumar et al., 1987, 1996; Dimasi-Theriou et al., 1993), while in others it may have an inhibitory effect (Chraibi et al., 1991; Pius et al., 1993; Burnett et al., 1994). Although the use of ethylene inhibitors in *in vitro* cultures has produced contradictory results (Krikorian, 1995), several reports (Perl et al., 1988; Hulme et al., 1992; Mollers et al., 1992) have demonstrated the suitability of STS as an inhibitor of ethylene action.

Rooting and Plant Development

Regenerated shoots were isolated and transferred to MS medium without growth regulators. Under these conditions, rooting occurred readily and, after 15–20 d, the percentage of rooted shoots reached 50–60%. Because this percentage was considered satisfactory, no further experiments were conducted on auxin-supplemented media. This result corroborates previous investigations with species of this genus (Moran Robles, 1978; Dornelas and Vieira, 1994; Kawata et al., 1995). However, Kantharajah and Dodd (1990) reported that naphthaleneacetic acid (NAA) was effective for root induction of *P. edulis* var. Norfolk Island.

The establishment of *in vitro*-grown plants in peat moss and perlite mix was easily achieved. Survival rate was 95% to 100%. After their acclimatization, the plants were transferred to a greenhouse and, finally, to the field, where they exhibited normal vegetative and floral development.

In conclusion, clonal micropropagation of *P. edulis* F. *flavicarpa* has been accomplished by organogenesis. To the best of our knowledge, this is the first study reporting the positive action of STS in *in vitro* cultures of *Passiflora* spp. Given the reduced morphogenic capacity of passionfruit leaves, the reported promotive effect of STS on shoot organogenesis may be useful when preparing micropropagation protocols for this economically important plant species.

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