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Effect of Morphological Factors, Antibiotics and *Agrobacterium* Co-cultivation in the Efficiency of Somatic Embryogenesis of Eggplant (*Solanum melongena* L.)

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Abstract

Induction of somatic embryogenesis from Brazilian eggplant variety F-100 was studied in response to four auxin types. NAA, at the optimal concentration of 54 μM, was the only one that resulted in the induction of somatic embryos in either leaf and cotyledon explant and, at much lower intensity and frequency, in hypocotyl and epicotyl explants. The optimal temperatures for embryo induction were 28 and 35℃ for cotyledon and leaf explants. Incubation at 22℃ caused a significant reduction both in the frequency and intensity of induction. This system was used to study the effects of position and orientation of the tissue on the culture medium as well as of antibiotics and explant co-cultivation with *Agrobacterium* on the efficiency of somatic embryo induction. The intensity of embryo induction was greater in the midsections of cotyledons relative to apical and basal regions, when the abaxial surface was in contact with the culture medium. The presence of antibiotics resulted in approximately 40-60% reduction of embryo induction relative to control explants, which originated 335±26.6 embryos. Co-cultivation with *Agrobacterium* before treatment with antibiotics caused a more drastic reduction (80-99%). Ampicilin treatment after cocultivation with *Agrobacterium* caused the least inhibitory effect, allowing the production of 60 embryos/explant.

Introduction

Eggplant is considered as a good system to investigate plant growth and development *in vitro* (Gleddie et al., 1986; Sharma and Rajam, 1995b; Momiyama et al., 1995). By applying available efficient protocols for *in vitro* regeneration (Sharma and Rajam, 1995a; Saito and Nishimura, 1994; Magioli et al., 1998) and genetic transformation (Rotino and Gleddie, 1990; Fári et al., 1995; Magioli et al., 2000), eggplant can also be specially suitable for gene regulation studies.

Several protocols have been described for inducing somatic embryogenesis from eggplant. The first one was reported by Yamada et al. (1967), who induced somatic embryogenesis from zygotic embryos cultured on MS supplemented with IAA. After that, several investigators described somatic embryogenesis induction from different explant types of several genotypes, mainly in response to NAA or 2,4-D. Gleddie et al. (1983) induced somatic embryogenesis from leaf explants cultured in the presence of 10 mg/L NAA, whereas Fillippone and Lurquin (1989) obtained best results from leaf and cotyledon explants cultivated in the presence of 1 mg/L NAA. On the other hand, Rao and Sing (1991) cultivated leaf explants in the presence of 0.1 or 2 mg/L NAA and failed to originate

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embryogenic callus, which were obtained in the presence of 8 mg/L NAA plus 0.1 mg/L kinetin. Saito and Nishimura (1994) reported higher induction rates from leaf and cotyledon explants using 50 μM 2,4-D as compared to the response induced by 10 mg/L NAA. Taken together, these observations demonstrate that the embryogenic response in eggplant is highly influenced by explant type and genotype as well as by auxin type and concentration.

F-100 is a widely cultivated eggplant variety in Brazil because of its agronomically important characteristics, such as high uniformity and productivity. It also exhibits a long productive period, as well as pathogens resistance. We used this variety as a model to study the embryogenic potential of eggplant, testing the influence of various auxin types and concentrations, explant sources and incubation temperatures. In addition, we also examined the embryogenic competence of cotyledon explants as influenced by the morphological position of explant on the donor plant as well as by its orientation on culture medium.

Although genetic transformation has been achieved with regeneration systems based on organogenesis (Fári et al., 1995; Magioli et al., 2000), the use of embryogenic systems for eggplant transformation via *Agrobacterium* has been reported to result in little or no success (Fillipone and Lurquin, 1989; Fári et al., 1995). Accordingly, attempts to use the above embryogenic system for eggplant transformation resulted only in callus formation and failed to originate transformants. As bactericidal antibiotics (Holford and Newbury, 1992; Sarma et al., 1995; Ling et al., 1998) and co-cultivation of explants prior to regeneration with *Agrobacterium* (Metz et al., 1995)have been shown to affect *in vitro* regeneration, we also examined the effects of these parameters on the intensity of somatic embryo production from cotyledon explants.

Material and Methods

Plant material and culture conditions

Seeds of eggplant (F100) were obtained from Agroceres Ltda. and stored at 4 C. The seeds were washed in distilled water containing 0.02% Tween 80, surface-sterilized for 25 min in a 5% sodium hypochlorite solution, rinsed three times in sterile distilled water and inoculated on half-strength MS basal medium (Murashige and Skoog, 1962) supplemented with 1.5% (w/v) sucrose and 0.7% agar (SIGMA).

Media pH was adjusted to 5.8 with 1 N NaOH prior to autoclaving (12℃ for 15 min) and after addition of growth regulators. Antibiotics were filter-sterilized before adding to the autoclaved medium. Seeds and explants were cultured on 25 mL of medium in 250 mL flasks (10 seeds or 5 explants per flask). Cultures were maintained in a growth chamber at 28±2℃ under a 16 hr photoperiod regime provided by cool-white fluorescent lamps (G&E) with a photon fluency of 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Establishment of embryo cultures

Leaf and cotyledon segments (50 mm^2) as well as hypocotyl and epicotyl explants (5 mm long) were excised from 21-day-old seedlings and cultured on MS medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4D) (4.5 and 22.6 μM), β-naphthoxyacetic acid (NOA) (5, 25 and 50 μM), picloram (PIC) (4 and 8 μM) or α-naphthaleneacetic acid (NAA) (5.4, 27 and 54 μM).

The effect of temperature was determined by incubating cultures under three different conditions (22, 28 or 35± 2℃). The effect of explant position on somatic embryo induction was evaluated by examining the response of different regions of cotyledons in addition to orientation on the culture medium. Explants were excised from the apical, median and basal regions of cotyledons and inoculated either with the abaxial or the adaxial side in contact with the culture medium. To induce conversion to whole plants, cotyledonary embryos were transferred to half-strength MS plus 1.5% sucrose and solidified with 1% phytagel (Saito and Nishimura, 1994). After 20 days on this medium, plantlets derived from these embryos were acclimatized for 2 weeks in a phytotron chamber and transferred to greenhouse.

Twenty explants were analyzed in each experiment and the treatments were repeated at least twice. The percentage of responsive explants and the number of embryos per explant were evaluated after 30 days of culture. Mean confidence intervals were given for α=0.05 using the *t*-test.

Effect of antibiotics and cocultivation with *Agrobacterium*

Cotyledon explants were dipped in an overnight culture of the disarmed strain *Agrobacterium* C58C1 (pMP90), blotted dry onto sterile filter paper, transferred to embryo induction medium and maintained in the dark for 48 hr. Alternatively, explants were co-cultivated for 48 hr in 10 mL liquid MS medium containing 100 μL of the bacterial suspension. After cocultivation or immediately after excision, explants were transferred to embryo induction medium (MS supplemented with 54 μM NAA) plus

various concentrations of one of the following antibiotics: cefotaxime (200, 300, 400 and 500 mg/L), ampicillin (300, 400 and 500 mg/L) or vancomycin (300, 400 and 500 mg/L). Two sets of control explants were used: (1) explants placed in embryo induction medium immediately after excision and (2) explants maintained in co-culture medium without *Agrobacterium* for 48 hr before transfer to embryo induction medium.

Results and Discussion

Establishment of embryo cultures

We examined the effects of various auxin types (NAA, 2,4-D, NOA and PIC) and concentrations on induction and development of somatic embryos of eggplant. Calli induced by 2,4-D and PIC showed low growth rates and were totally oxidized after a period of 20 days of culture. NOA induced the formation of friable rhizogenic calli at the concentrations of 5 and 25 μM (data not shown). Although all auxins studied promoted the development of friable calli from all explant types, NAA was the only auxin that yielded sustainable embryogenic calli which was similar to an observation made by Gleddie et al. (1983). While there has been success in embryo induction with auxins such as 2,4-D in other eggplant cultivars (Alicchio et al., 1982; Saito and Nishimura, 1994), F100 variety seems to be hypersensitive to 2,4D probably as a consequence of genotypic factors. That may be why several other reports have found NAA to be useful for studies on somatic embryo formation in eggplant (Matsuoka and Hinata, 1979; Fobert and Webb, 1988; Rao and Singh, 1991; Gleddie et al., 1993; Sharma and Rajam, 1995a).

The frequency of embryonic callus production varied with type of organ and concentration of NAA. Cotyledon and leaf explants showed higher frequencies and we observed a proportional increase in embryonic competence relative to auxin concentration. In contrast, hypocotyl and epicotyl explants showed the lower frequencies with maximal response up to 27 μM NAA (Figure 1A). The intensity of embryo induction was also influenced by explant type and auxin concentration. Both the hypocotyl and epicotyl explants demonstrated relatively low competence to all NAA concentrations examined. Cotyledon and leaf explants displayed greater competence relative to the other tissues and also showed a linear increase in embryo induction with increasing concentrations of NAA (Figure 1B), consistently with the results reported by Alicchio et al. (1982). On the other

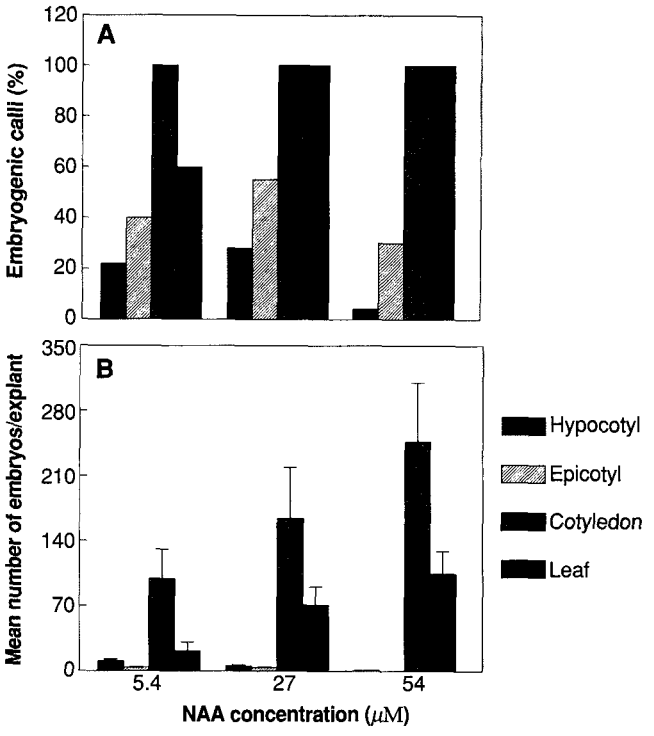

Figure 1. Percentage of embryo-producing explants (A) and mean number of embryos per explant (B) from different tissues after 30 d of culture on MS medium supplemented with NAA. Bars represent ± SE.

hand, these results are in contrast to those reported by Sharma and Rajam (1995a) who found the poorest embryonic response in hypocotyls but no differences between leaf and cotyledon explants.

We observed that incubation temperature is a crucial parameter for both frequency and intensity of embryo production. Optimal induction rates were similar for leaf and cotyledon explants at 28 and 35°C, in accordance to those reported previously with leaf explants (Gleddie et al., 1983) and isolated microspores (Miyoshi, 1996). In addition, we demonstrated that embryogenic competence is greatly reduced at 22°C and that this reduction is influenced by explant type (Figure 2). Whereas regeneration frequency and intensity from cotyledons is reduced by 25% and 52.4% respectively, leaf explants response was reduced by 70% and 72%.

Transfer of mature embryos to MS medium (either basal or half strength) solidified with 0.7% agar resulted in a poor conversion into plants and development of callus. These results are in accordance with those of other authors who reported limited conversion of somatic embryos into plantlets due to abnormalities such as hyperdricity, lack of apical meristem, cotyledon fusions and inefficient

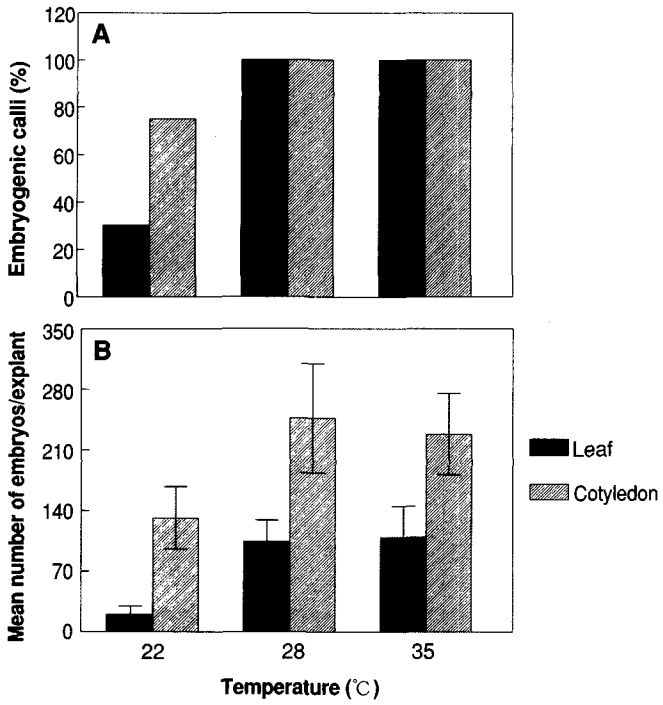

Figure 2. Effect of culture temperature on somatic embryo induction from leaf and cotyledon explants after 30 d of culture on MS medium supplemented with 54 μM NAA. Bars represent ± SE.

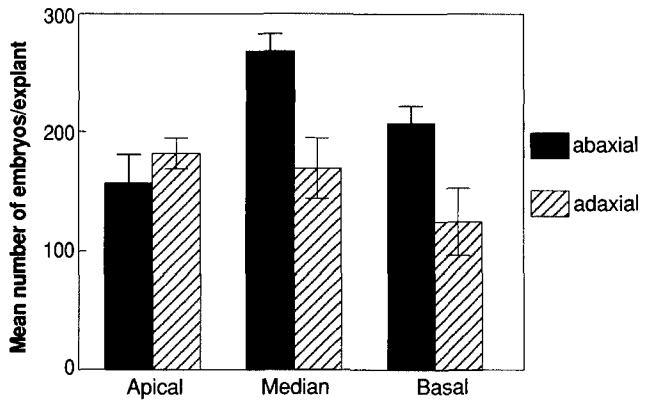

Figure 3. Effect of the original position of explants on the donor organ and explant orientation on the culture medium in somatic embryo production intensity. Explants were obtained from the apical, median and basal regions of cotyledons. All explant types were placed with either the abaxial or the adaxial side in contact with the culture medium. Bars represent ± SE.

maturation (Gleddie et al., 1983; Saito and Nishimura, 1994). The use of ABA to reduce the frequency of abnormal embryos (Ammirato, 1983; Von Arnold and Hakman, 1988; Goebel-Tourand et al., 1993) or of GA_3 to induce embryo-to-plant conversion were either unsuccessful or resulted in very low rates of whole plant development (data not shown). High conversion rates of embryos into phenotypically normal plants (92%) were only achieved when embryos at the cotyledonary stage were cultured on half-strength MS medium solidified with 1% phytagel (Saito and Nishimura, 1994).

Despite the intense somatic embryo induction from cotyledon explants cultured in the presence of 54 μM NAA, the response of individual explants was very heterogeneous, resulting in high standard error values (Figures 1 and 2). In order to circumvent this problem, we studied the effect of morphological position of the explants on the intensity of embryo induction by cutting cotyledons into segments that represented areas from the apical to the basal portion of each. Each of these was placed on induction medium with either the ad or abaxial surface in contact with the medium. When cultured with the abaxial surface in contact with the culture medium, the middle section of the cotyledon displayed a significantly ($p<0.05$) greater embryogenic competence relative to the other regions (Figure 1). These results are in contrast with the report by Gleddie et al. (1983), in which explant orientation on the medium was found to influence organogenesis but not embryogenesis. In addition, the mean number of embryos/explant produced by individual segments displayed low standard deviations values (267.6 ± 15.5 embryos/explant), resulting in a more homogeneous response than that obtained when the three segments were indiscriminately pooled (247 ± 63.0 embryos/explant).

The different responses displayed by the three cotyledon segments may have resulted from a gradient of phytohormones (Ulvskov et al., 1992). Polyamine content, distribution and metabolism have also been correlated with position effects on the embryogenic competence in eggplant (Fobert and Webb, 1988; Sharma and Rajam, 1995a; 1995b; Yadav and Rajam, 1997; Yadav and Rajam, 1998).

Effect of antibiotics and co-cultivation with *Agrobacterium*

In recent years, there has been increasing awareness of the effect of bactericidal antibiotics stimulating (Ling et al., 1998) or inhibiting (Sarma et al., 1995; Ling et al., 1998) the regenerative potential of explants, depending on each specific combination between a given antibiotic and plant species. In the present work, we studied the effect of three antibiotics representing different groups: ampicillin (penicillins), cefotaxime (cephaloporins) and vancomycin

(glycopeptides). All of them are inhibitors of the bacterial cell wall synthesis.

We found that callus formation and development were not affected in the presence of effective lethal concentrations usually adopted in transformation protocols to eliminate *Agrobacterium* (200, 400 and 500 mg/L for cefotaxime, ampicillin and vancomycin, respectively). However, antibiotic treatment reduced the number of embryos produced per callus by 52% (cefotaxime and ampicillin) or 37% (vancomycin) relative to control explants. Co-cultivation with *Agrobacterium* for 48 hr in liquid MS medium prior to antibiotic treatment caused a more drastic reduction in the intensity of embryo production. Intensity of embryo production from co-cultivated explants was most severely depressed in the presence of vancomycin and cefotaxime and least depressed in the presence of ampicillin (Table 1). No significant differences in embryo production were observed between the two sets of control explants used. The presence of MES in the co-cultivation medium in order to avoid acidification did not have any significant effect in this inhibition. Similarly, the presence of the antioxidants Polyvinylpirrolidone (PVP) (0.25%) and Dithiotreiol (DTT) (2.5 mg/L) (Perl et al., 1996) during co-cultivation and in the induction medium did not reduce the inhibitory effects (data not shown).

Table 1. Effect of bactericidal antibiotics and *Agrobacterium* in the efficiency of somatic embryogenesis from cotyledon explants.

Treatment	Antibiotics	Concentration (mg/L)	No. of embryos/callus[a]
Antibiotics	Vancomycin	300	140.7 ±29.7
		400	174.5 ±27.4
		500	203.3 ±39.9
	Ampicillin	300	192.8 ±15.3
		400	158.2 ± 7.3
		500	148.5 ±25.7
	Cefotaxime	200	159.8 ±17.7
		300	104.5 ± 7.4
		400	147.7 ±13.5
		500	92.31± 6.7
Cocultivation followed by antibiotics	Vancomycin	300	*
		400	*
		500	1.7 ± 0.2
	Ampicillin	300	*
		400	59.5 ±13.1
		500	26.8 ±10.1
	Cefotaxime	200	14.3 ± 6.4
		300	7.5 ± 2.0
		400	12.0 ± 3.6
		500	5.2 ± 1.0
Untreated control	-	-	335.0 ±26.6

[a]Mean ± standard error.
*Non-lethal concentration.

Although there is limited information concerning the mode of action of antibiotics in plants, their effects on *in vitro* regeneration are apparently due to hormone-like properties. For example, Holford and Newbury (1992) demonstrated that carbenicillin breakdown originates phenylacetic acid, which exhibits auxin activity. It is also known that the same antibiotics may have stimulatory or inhibitory effects, depending on the concentration used. In general, low concentrations of cefotaxime and carbenicillin stimulate morphogenesis whereas high concentrations cause an inhibitory effect (Nauerby et al., 1997). In the present study, it is possible that the high concentrations of antibiotics required to eliminate *Agrobacterium* caused a hormone misbalance (Sarma et al., 1995) which resulted in the reduction of embryogenic competence.

The necrotic response caused by *Agrobacterium* cocultivation has been correlated with reduced shoot regeneration (Metz et al., 1995) and transformation efficiency (Lloyd et al., 1986; Babic et al., 1998) of other species. The data presented in this report indicate that *Agrobacterium* also interferes with the embryogenic competence of eggplant and that the specific combination between *Agrobacterium* and antibiotics may induce different inhibition levels. The reduction caused by *Agrobacterium* co-cultivation plus ampicillin treatment corresponds to 62.5% less then the inhibition promoted just by the treatment with the antibiotic and to 82.2% less than that observed in control explants. Interestingly, no significant differences ($p<0.05$) were observed among the inhibitory levels of the different antibiotics in non-infected explants. The reduction of embryogenic competence caused by antibiotics and *Agrobacterium* may explain our and other investigators (Fillipone and Lurquin, 1989) difficulty to transform as well as the low transformation efficiency observed by Fári et al. (1995) when embryogenic systems were used in *Agrobacterium*-based protocols.

Rotino and Gleddie (1990) reported that *Agrobacterium* co-cultivation delayed the onset of shoot regeneration from eggplant leaf explants by about 4-8 weeks and it is possible that *Agrobacterium* would cause a similar effect in somatic embryogenesis. In our system, analysis of embryo development has revealed that histological alterations which indicate expression of the embryogenic program begin as early as two days after culture initiation. In addition, it was observed that after the first week of

culture root-like structures emerge from the same cell types which originated the somatic embryos and that at the same time, the intensity of embryo formation is strongly reduced (data not shown). Thus, the inhibitory effects of antibiotics and *Agrobacterium* on somatic embryo formation may be the result of the interaction between the physiological alterations caused by these treatments and the delicate processes of gene regulation which are induced in early culture stages.

In conclusion, we clearly demonstrated that the recovery of transgenic eggplant using an embryogenic system would require the use of ampicillin instead of cefotaxime, which is the most used antibiotic to eradicate *Agrobacterium*. Our data corroborate the idea that an initial screening for antibiotic sensitivity is a fundamental step in establishing new transformation protocols.

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