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CALLUS INDUCTION AND PLANT REGENERATION OF TOMATO (*Lycopersicon esculentum* CV. IPA 5) VIA ANTHER CULTURE

INDUÇÃO DE CALOS E REGENERAÇÃO DE PLANTAS A PARTIR DO CULTIVO *IN VITRO* DE ANTERAS DE TOMATE (*Lycopersicon esculentum* CV. IPA 5)

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SUMMARY

Different growth regulators combinations were tested on the production of anther callus in tomato cultivar IPA 5. Calli were induced on media supplemented with 1.0mgL^{-1} gibberellic acid (GA_3), 0.05mgL^{-1} α-naphthaleneacetic acid (NAA) plus 0.1mgL^{-1} 6-benzylaminopurine (BAP), or with 1.0mgL^{-1} BAP plus 1.0mgL^{-1} NAA. The medium containing 1.0mgL^{-1} BAP and 1.0mgL^{-1} NAA produced the highest calli frequency, and promoted plant regeneration by indirect organogenesis, when calli were transferred to 0.01mgL^{-1} BAP and 0.001mgL^{-1} NAA. Plants regenerated presented tetraploid cells and rare diploid cells. These tetraploid plants could be used as source for further obtainment of trisomic lines, for the purpose of genic localization studies and protein compounds analysis.

***Key words**: growth regulators, organogenesis, tetraploid cells.*

RESUMO

Diferentes combinações de reguladores de crescimento foram testadas na produção de calos a partir do cultivo de anteras de tomate cultivar IPA 5. Calos foram induzidos no meio suplementado com 1,0mgL^{-1} de ácido giberélico (GA_3) + 0,05mgL^{-1} de α-ácido naftalenoacético (ANA) + 0,1mgL^{-1} de 6-benzilaminopurina (BAP), ou com 1,0mgL^{-1} de BAP + 1,0mgL^{-1} de ANA. O meio contendo 1,0mgL^{-1} de BAP e 1,0mgL^{-1} de ANA produziu a maior freqüência de calos e promoveu a regeneração de plantas através de organogênese indireta, quando os calos foram transferidos para 0,01mgL^{-1} de BAP e 0,001mgL^{-1} de ANA. As plantas regeneradas apresentaram células tetraplóides e, raramente, células diplóides. Estas plantas tetraplóides podem servir como fonte para posterior obtenção de linhagens trissômicas, para serem utilizadas em estudos de localização gênica e em análises de compostos protéicos.

***Palavras-chave**: reguladores de crescimento, organogênese, células tetraplóides.*

INTRODUCTION

Plant tissue culture techniques are recognized as useful instruments in crop improvement. Among these techniques, ***in vitro*** anther culture stands out and is an increasingly powerful tool when integrated into breeding programs (HU & ZENG, 1984). This technique allows the acceleration of plant breeding by providing homozygous doubled haploids within a comparatively short time (NURHIDAYAH ***et al.***, 1996). In addition, obtaining haploid plants from segregant generations facilitates genetic analysis, eliminating the complexity of the heterozygous state (MORAES-FERNANDES, 1990).

Several studies have been reported with tomato anther culture. However, the function carried out by factors such as microspore developmental stages (GULSHAN ***et al.***, 1981; SUMMERS ***et al.***, 1992), dark-light regimes (JARAMILLO & SUMMERS, 1991), and growth regulators (GRESSHOFF & DOY, 1972; ROGOZINSKA & SKUTNIK, 1974) are still unclear.

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SHARP ***et al.*** (1971) are among the pioneers of ***in vitro*** tomato anther culture. They observed that exogenous growth regulators have no obvious effect on the induction of calli proliferation. On the other hand, GRESSHOFF & DOY (1972) observed that differentiation of haploid calli of ***L. esculentum*** can be controlled by hormones added to a defined medium. Other authors also noticed different responses to distinct dosages of growth regulators on the formation of androgenic calli (DAO & SHAMINA, 1978; GULSHAN ***et al.***, 1981). In a work by ZIV ***et al.*** (1982), doubled haploid plants were regenerated from non-allelic male-sterile mutants (***ms*** 10^{35}), using a medium containing 0.5mgL^{-1} indole-3-acetic acid (IAA) and 0.25mgL^{-1} zeatin. Although, the regeneration of tomato plants from microspores has been strongly dependent on this mutant genotype.

The purpose of this work was to study the effect of different growth regulators on ***in vitro*** induction and growth of tomato anther calli, and their later differentiation into plants.

Table 1 - Growth regulators tested on anther calli formation (basal medium according to CAPPADOCIA & SREE RAMULU, 1980)

Concentration of growth regulators (mg L^{-1})				
Medium 1[1]	Medium 2[2]	Medium 3	Medium 4	Medium 5
1.0 GA_3 0.05 BAP 0.1 NAA	1.0 BAP 1.0 NAA	2.0 2,4 D[3]	0.1 TIBA[4]	0.5 TIBA[4]

[1] Growth regulators used by CAPPADOCIA & SREE RAMULU (1980).
[2] Growth regulators recommended by ROGOZINSKA & SKUTNIK (1974).
[3] Auxin recommended by HENDER (1973).
[4] Anti-auxin used by WILLADINO ***et al.*** (1995), in maize anther culture.

MATERIALS AND METHODS

Tomato plants of cultivar IPA 5 (2n=24) were grown in a greenhouse, watered daily and fertilized once a week. Flower buds 5 to 6mm long were harvested. The developmental stage of anthers varied from the beginning of meiosis to tetrad. For cytological analysis, the anthers were fixed in Carnoy (3:1) and stained in 1% (w/v) hematoxylin as described by FUJII & GUERRA (1998).

The buds harvested in the morning were conditioned in a moist chamber and submitted to a cold pretreatment at 5^{o}C for 6h, in the dark. Later, they were surface sterilized in 70% (v/v) ethanol, for 15s, immersed in a 0.5% (v/v) sodium hypochlorite solution for 5min and then rinsed three times in sterile distilled water. Anthers were removed aseptically and plated on five callus induction media varying the growth regulator's composition (table 1). Basic medium, according to CAPPADOCIA & SREE RAMULU (1980), containing MURASHIGE & SKOOG (1962) salts (MS), myo-inositol, glycine and vitamins of NITSCH (1969) medium, 20% (w/v) sucrose and 0.7% (w/v) agar (pH 5.8) was used for all treatments. The (10cm) Petri dishes used for culture were maintained in the darkness in a growth-chamber at 26 ± 1^{o}C for one week and, afterwards, exposed to 16h light photoperiod (90 – 110µmolm^{-2}s^{-1}) provided by fluorescent illumination. The frequency of anthers producing calli was recorded after 30 and 60 days.

The calli produced were transferred to a regeneration medium composed of the same basal medium supplemented with 0.01mgL^{-1} 6-benzylaminopurine (BAP) and 0.001mgL^{-1} α-naphthaleneacetic acid (NAA). The calli with organogenic characteristics were partially fixed in formolin/ glacial acetic acid/ 50% ethanol (5:5:90 v/v/v), for posterior histological analysis, as described by JOHANSEN (1940). In order to identify the ploidy level, root tips of regenerated plants were pretreated with 0.002M 8-hydroxyquinoline for 24h at 10^{o}C, fixed in Carnoy (3:1), hydrolyzed in 5N HCl for 20min, squashed in 45% (v/v) acetic acid and stained with 2% (v/v) Giemsa (GUERRA, 1983).

The experiment was entirely randomized, with five treatments (six replications per treatment), where the experimental unit consisted of a Petri dish containing 25 anthers, performing 150 anthers per treatment. Statistical analyses were carried out by the ANOVA and Tukey test, at a 1% probability level, using $\sqrt{x+0.5}$ transformation.

RESULTS AND DISCUSSION

Calli formation was observed exclusively in media containing both auxins and cytokinins (1.0mgL^{-1} GA_3 plus 0.05mgL^{-1} BAP plus 0.1mgL^{-1} NAA, medium one; 1.0mgL^{-1} BAP plus 1.0mgL^{-1} NAA, medium two). A significant increment in callus production was verified as time progressed, for both media (table 2). Medium two presented the

Table 2 - Growth regulators composition affect the average media and percentage of the number of anthers that produced calli.

	30 days		60 days	
Growth regulators (mg L^{-1})	Average	%	Average	%
1.0 BAP + 1.0 NAA (medium 2)	4,0 a B	16	16,8 a A	67,2
1.0 GA_3 + 0.05 BAP + 0.1 NAA (medium 1)	2,8 a B	11,2	6,0 b A	24
2.0 2,4 D (medium 3)	0 b	0	0 c	0
0.1 TIBA (medium 4)	0 b	0	0 c	0
0.5 TIBA (medium 5)	0 b	0	0 c	0

[1] Values in a column followed by different lower case or in a line followed by different capital letter are significantly different at the 0.01 probability level according to a Tukey test.

highest frequency of calli formation: 16% after 30 days and 67% after 60 days.

For some members of Solanaceae family, the inclusion in the culture medium of an auxin alone, or in combination with a cytokinin, frequently induces the formation of pollen calli (CANHOTO ***et al.***, 1990). The importance of this hormonal balance in tomato anther culture has been described by several authors. GULSHAN ***et al.*** (1981) verified that the medium containing 2.0mgL^{-1} NAA and 1.0mgL^{-1} kinetin was the most efficient for callus formation. This same auxin/ cytokinin (2/1) proportion, used in the present experiment (0.1mgL^{-1} NAA plus 0.05mgL^{-1} BAP) increased callus production significantly, but the highest frequency was observed in a medium supplemented with a 1/1 mix of auxin/cytokinin. This result is in agreement with ROGOZINSKA & SKUTINK (1974), who used both NAA and BAP at a concentration of 1.0mgL^{-1}.

In this experiment, auxin in the form of 2,4 dichlorophenoxyacetic acid (2,4 D) (2.0mgL^{-1}) did not stimulate callus induction. However, HENDER (1973) verified better callus development when cultivating tomato anthers in this same concentration of 2,4 D. The presence of 2,3,5 triiodobenzoic acid (TIBA) in the culture (medium four and five) did not propitiate either calli production, although this anti-auxin has been used to obtain doubled haploid maize (WILLADINO ***et al.***, 1995).

In the medium supplemented with 1.0mgL^{-1} GA_3 plus 0.05mgL^{-1} BAP plus 0.1mgL^{-1} NAA, a darkening of the anthers surface was observed, although green calli were produced in about 21% of them. GRESSHOFF & DOY (1972) also observed a similar process of calli formation, but these calli presented a pale yellow color. Green calli were also produced on the medium supplemented with 1.0mgL^{-1} BAP and 1.0mgL^{-1} NAA.

Histological analysis showed that some of these calli presented tracheids, vessel elements and phloem cells initiation. Such structures were verified by GULSHAN ***et al.*** (1981) in tomato anther callus and the initiation of vessel elements was favoured by addition of NAA plus kinetin and that of the phloem in the presence of 2,4 D plus BAP.

The calli that presented development of vascular elements were subcultured on a medium containing 0.01mgL^{-1} BAP plus 0.001mgL^{-1} NAA, showing later organogenic potential. After five months, plant regeneration had occurred on 0,27% of the calli obtained (figure 1a). Cytological analysis showed that these plants presented tetraploid cells and rare diploid cells (figure 1b). Such differences on ploidy level can be induced by components of the culture medium, time of culture, explant source, and the plant genotype (KARP, 1988). This instability was observed by KORNNEEF ***et al.*** (1989) in cells of plants regenerated from leaves of haploid genotypes of tomato. Of the 149 metaphase cells of root tips that were analyzed 41% were haploid, 58% diploid and 1% tetraploid. ANCORA ***et al.*** (1977), studying genotypes ***L. peruvianum***, also observed that cells from one anther callus can differ in ploidy level.

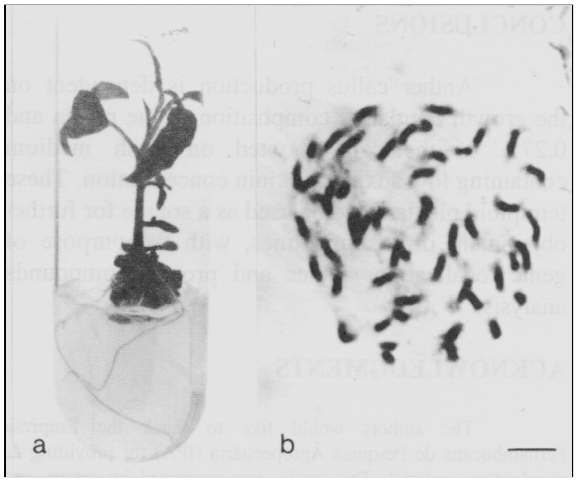

Figure 1 - (a) Regenerated plant obtained through indirect organogenesis. (b) Tetraploid cell (2n=4x=48) of the same plant. Bar in ***b*** represents 8.1mm for ***a*** and 5µm for ***b***.

Besides the microspores, growth of somatic tissue (filament, connective, or anther wall) could be induced (WENZEL & FOROUGHI-WEHR, 1984). LEVENKO ***et al.*** (1977) verified that anther wall cells took part in the formation of tomato mixoploid calli. CAPPADOCIA & SREE RAMULU (1980) and ZAMIR ***et al.*** (1980; 1981) obtained plants derived from anther culture. However they were originated from somatic cells.

In anther culture, there are two pathways for plant regeneration: direct or indirect androgenesis (CANHOTO ***et al.***, 1990). Most of the plants regenerated through direct embryogenesis are haploids, while plants obtained from calli are generally diploids (CONSTANTIN, 1981).

In the present study we described the induction of anther organogenic calli, and regeneration of tomato tetraploid plants. In ***Oenothera hookeri*** De Vries, diploid plants were also obtained through indirect androgenesis (MARTINEZ & HALAC, 1995). In Solanaceae family, as in ***Datura metel*** (IYER & RAINA, 1972), both kinds of androgenesis, direct (embryos formation) or indirect (embryos or shoots formation), may be observed (OGURA, 1990). It appears that the type of androgenesis followed is to some extent determined by the culture medium composition and by the endogenous levels of growth regulators prevailing within anther, varying the magnitude of these influences with the species considered (CANHOTO ***et al.***, 1990). Further studies are necessary to understand the pathway by which environmental and genetical factors induce shooting from callus tissue (MARTINEZ & HALAC, 1995).

CONCLUSIONS

Anther callus production is dependent on the growth regulators composition of the media and 0,27% of the calli shooted on fresh medium containing low auxin-cytokinin concentration. These tetraploid plants could be used as a source for further obtainment of trisomic lines, with the purpose of genic localization studies and protein compounds analysis.

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