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BRIEF COMMUNICATION

Influence of CCC, putrescine and gellam gum concentration on gynogenic embryo induction in *Allium cepa*

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Abstract

The induction of haploid plants by *in vitro* gynogenesis is a promising practice in onion breeding. In order to increase the frequency of embryo regeneration and haploid plant production in Valcatorce INTA, Cobriza INTA and Navideña INTA cultivars, putrescine and CCC were used, either as a component of the culture media or by spraying or injecting them to the umbels. Additionally, two concentration of gellam gum were tested. A higher number of gynogenic embryos was achieved by using 7 g dm^{-3} gellam gum, and this number was not affected by the addition of putrescine to the media. CCC sprayed at the umbels significantly increased the gynogenic embryo rate, which was more than three times higher than the control. Cobriza INTA showed the highest induced embryo rate (4.76 %).

Additional key words: growth regulators, haploid, onion.

The production of pure lines to produce F_1 hybrid in onion not only demands long time, between 6 to 10 years by selfcrossing, but also it is difficult due to inbreeding depression (Martínez *et al*. 2000). The induction of haploid plants by *in vitro* gynogenesis is a way to accelerate the production of homozygous lines in onion. The use of flower culture to generate doubled-haploid (DH) homozygous lines and further production of F_1 hybrid by crossing the homozygous parents, has become a promissory practice in onion breeding.

Successful regeneration of haploid plants has been reported by several authors (Martínez *et al.* 2000, Hassandokht and Campion 2002, Alan *et al.* 2004). The efficiency of this technique depends on the genotype, media composition and flower bud development (Bohanec 2002). Muren (1989) showed that parthenogenic response is highly affected by the donor plant genotype, while others authors reported that the gynogenic response of onion accessions was extremely dependent on their geographic origin (Geoffriau *et al.* 1997, Bohanec and Jakše 1999, Hassandokht and Campion 2002). Taking into account Bohanec *et al.* (2003), Argentinean genotypes could be categorized as a very low responsive material (Martínez *et al*. 1995, 2000). The media composition is also an important factor; improved responses have been favored by high sucrose concentration (Muren 1989) and supplements of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl-aminopurine (BA) (Campion *et al.* 1992, Bohanec and Jakše 1999). Other growth regulators like polyamines have been reported to be essential for growth and development of living tissues. Martínez *et al.* (2000) showed that putrescine and spermidine induced the onset of embryogenesis and increased the number of onion gynogenic embryos and plantlets obtained. The gelling agent is another factor that may contribute to the success of the technique. Jakše *et al*. (1996) reported that gellam gum (*Gelrite*) doubled the number of regenerated

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - benzylaminopurine; CCC - 2-chloroethyltrimethyl ammonium chloride.

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embryos, compared to agar solidified media, although the number of abnormal regenerants were higher with gellam gum. The flower bud development stage also affects the technique. Musial *et al.* (2001) suggested that embryo sacs at early stages of development might be more appropriate for haploid induction than mature megagametophytes, probably because immature embryo sacs are more competent for parthenogenesis. The most successful period to culture the flowers is between 3 to 5 d before anthesis, which corresponds to a flower-bud-size between 3 to 5 mm long (Alan *et al*. 2004), varying with the genotype the exact size to succeed (Michalik *et al.* 2000).

Due to the fact that Argentinean genotypes are considered to be low responsive gynogenic material, our goal was to increase the frequency of embryo regeneration and haploid plant production. In order to achieve this we propose the use of putrescine and CCC, either as a component of the culture media or by umbel spray or injection and different gellam gum concentration.

Experiments were carried out with cultivars Valcatorce INTA (Val), Cobriza INTA (Cob) and Navideña INTA (Nav) described by Galmarini (2000). Ninety bulbs of each cultivar were planted in May and grown using standard horticultural practice. Flowers were surface-sterilized with ethanol 96 % for a 1 min, then with 10 % sodium hypochlorite (60 g dm^{-3} active chlorine) containing few drops of *Tween 20* for 15 min and rinsed three times with sterile water. Thirty flowers were cultured in Petri dishes in a basal medium consisting of BDS macro, micro elements and vitamins (Dustan and Short 1977), 0.5 g dm^{-3} inositol, 0.2 g dm^{-3} proline and 100 g dm^{-3} sucrose. pH was adjusted to 6 before autoclaving. Three different experiments were performed in the same year.

In the first experiment, 30 onion plants were used for each cultivar. Three to five days before anthesis 3210, 3510 and 4320 flowers coming from differents umbels were pulled in one common batch for Val, Cob and Nav, respectively. They were cultured in basal medium supplemented with different growth regulators and gellam gum concentration (Table 1). Putrescine was added by sterile filtration.

In the second one, 30 inflorescence stalks from each cultivar were sprayed with 0.08 or 0.16 g dm^{-3} putrescine or 0.1 g dm^{-3} CCC when the umbel´s flowers began to bloom. Three to five days before anthesis, 1350, 1440 and 1470 flowers pulled in one common batch for Val, Cob and Nav were dissected and sterilized as described above and cultured in basal medium supplemented with 0.002 g dm^{-3} 2,4-D, 0.002 g dm^{-3} BA and 3 g dm^{-3} gellam gum.

In the third one, 12 inflorescence stalks from Nav and Val cultivars were injected with 40 cm^3 of 0.016; 0.16 or 1.6 g dm^{-3} putrescine when the umbel´s flowers began to bloom. Three to five days before anthesis flowers were dissected one day before treatments (control) and every 48 h during 10 d. They were sterilized and placed in basal medium plus 0.002 g dm^{-3} BA.

In all the experiments the flowers were left on these media until the sprouting of the embryos, which then were transferred to a half strength basal medium supplemented with 30 g dm^{-3} sucrose. The culture conditions were 16-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1}, *Philips* cool-white fluorescent tubes) and day/night temperature of 25/20 ± 2 °C. Regeneration rates, number of haploid and normal plantlets were scored. Data were analysed by comparison of proportions using the *Statgraph plus vs. 4* software.

The ploidy level of normal plantlets was analyzed by flow cytometry (*Partec PAS,* Ljubljana, Slovenia) as previously described by Bohanec and Jakše (1999).

Martínez *et al*. (2000) improved the induction rate of gynogenic onion embryo using polyamines in a two-step culture procedure. The present report evaluated the combination of putrescine with or without BA in a one step procedure. In cv. Cob the number of gynogenic embryos was significantly increased in medium containing 0.64 g dm^{-3} putrescine, 0.002 g dm^{-3} BA and 7 g dm^{-3} gellam gum (2.5 %) and also in medium containing 0.002 g dm^{-3} 2,4-D, 0.002 g dm^{-3} BA and 7 g dm^{-3} gellam gum (1.48 %) (Table 1). The results obtained for this genotype, confirmed also that the medium

Table 1. Effect of media composition [g dm^{-3}] on regeneration of gynogenic embryos from flowers of onion cvs. Cob, Nav and Val. Different letters in the column indicate significant differences between treatments for each cultivar ($P \leq 0.05$).

Putrescine	BA	2,4-D	Gellam gum	Cob cult. flowers	regenerants [%]	Nav cult. flowers	regenerants [%]	Val cult. flowers	regenerants [%]
0.32	-	-	3	300	0.00 a	90	0.00 a	330	0.00 a
0.32	-	-	7	390	0.51 a	510	0.59 a	270	0.00 a
0.32	0.002		3	270	0.00 a	240	0.42 a	420	0.71 a
0.32	0.002		7	120	0.83 a	570	1.22 a	90	0.00 a
0.64	-	-	3	360	0.00 a	270	0.00 a	480	0.21 a
0.64	-	-	7	360	0.00 a	720	0.00 a	300	1.00 a
0.64	0.002		3	270	0.00 a	210	0.00 a	240	0.42 a
0.64	0.002		7	120	2.50 b	240	0.83 a	270	0.00 a
-	0.002	0.002	3	180	0.00 a	150	0.00 a	210	0.47 a
-	0.002	0.002	7	270	1.48 b	480	1.46 a	240	1.67 b

supplemented with putrescine and BA offers an alternative to the most frequently used hormone combination. In cv. Val the medium supplemented with 2,4-D, BA and the highest gellam gum concentration showed the maximum percentage of regenerants (1.67 %), while cv. Nav did not show any significant differences among treatments. Therefore, each genotype require specific medium for highest embryos production.

Table 2. Effect of gellam gum concentration on regeneration of gynogenic embryos from flowers of onion cvs. Cob, Nav and Val cultured in basal medium with different growth regulators. Different letters in the column indicate significant differences ($P \leq 0.05$).

Cultivar	Gellam gum [g dm^{-3}]	Cultured flowers	Regeneration [%] total regenerants	normal plantlets
Cob	7	1260	0.79 b	0.47
	3	1380	0.00 a	0.00
Nav	7	2520	0.75 b	0.04
	3	960	0.10 a	0.10
Val	7	1170	0.59 a	0.43
	3	1680	0.36 a	0.12

Table 3. Effect of putrescine and CCC spray on the gynogenic embryo regeneration from flowers of onion cvs. Cob, Nav and Val cultivated in basal medium plus 0.002 g dm^{-3} 2,4-D, 0.002 g dm^{-3} BA and 3 g dm^{-3} gellam gum. Different letters in the column indicate significant differences between treatments for each cultivar ($P \leq 0.05$).

Cultivar	Treatment [g dm^{-3}]	Cultured flowers	Total reg. [%]	Normal plantlet [%]
Cob	putrescine 0.08	270	1.85 b	1.10
	putrescine 0.16	330	0.00 a	0.00
	CCC 0.1	210	4.76 c	4.76
	control	150	1.33 b	0.00
Nav	putrescine 0.08	270	0.00 a	0.00
	putrescine 0.16	300	0.00 a	0.00
	CCC 0.1	120	0.83 a	0.00
	control	330	0.00 a	0.00
Val	putrescine 0.08	390	0.51 b	0.29
	putrescine 0.16	210	0.00 a	0.00
	CCC 0.1	240	2.91 c	1.25
	control	240	0.83 b	0.42

Cob and Nav exhibited the highest rate with 7 g dm^{-3} gellam gum, which statistically differed with the lower concentration of the gelling agent (Table 2). However, Val was not affected by the gellam gum concentration. Jakše *et al.* (1996) reported high number of regenerated embryos when they used gellam gum solidified media, but also high number of abnormal regenerants. In the present study, Nav showed a high number of abnormal regenerants when the concentration of the gellam gum was increased. Nevertheless, in Cob cultivar the rise in the gellam gum concentration enhanced the embryo regeneration, and half of them developed into normal plantlets (Table 2). It will be necessary to determine the suitable concentration of the gelling agent for each cultivar since the response depends on the genotype.

CCC significantly increased the gynogenic embryo rate in Val and Cob, which were more than three times higher than the control (Table 3). In a previous work, similar results were obtained in Nav and Torrentina genotypes, when CCC was added to the storage solution where the cut umbels were soaked (Martínez, unpublished). Since the immature embryo sac stage is considered optimal for induction of gynogenic embryos, as was suggested by Musial *et al.* (2001), the plant growth retardant CCC could delay the conversion from immature to mature embryo sac stage and prolong the contact time with the induction media, and so the probability for the gynogenic induction. This fact could explain the Cob and Val behaviour. Cob showed the highest embryo rate (4.76 %) reported, so far, in an Argentinean onion. Sixty one percent of the regenerated embryos developed into normal plantlets. The lowest rate of gynogenic embryos was achieved by Nav, exhibiting a high number of abnormal plantlets.

Although Martínez *et al.* (2000) demonstrated a positive effect of polyamines on gynogenic embryo yield when they were added to the culture media, in the present work, putrescine sprays did not stimulate the regeneration embryo rate in any of the cultivars when it was used at 0.08 g dm^{-3} (Table 3) and the higher concentration (0.16 g dm^{-3}) showed an inhibitory effect, probably through the oxidation products from this polyamine. More experiments are necessary to determine the optimal time and concentration of putrescine sprays to the umbels.

The injection of putrescine at the low concentration (0.016 g dm^{-3}) allowed a significantly higher production of gynogenic embryo in Nav and Val, although the latter genotype did not show significant differences from the control. The medium concentration (0.16 g dm^{-3}) yielded lower percentage of regeneration than the higher concentration although it was significantly superior to the control for Nav cultivar. The effect of putrescine at the highest concentration seemed to be detrimental in both genotypes (Table 4). These results are similar to those mentined above. Isolating the flower 6 d after the polyamine injection gave the best result (data no shown) in both cultivars. Only 2 from 12 donor plants were able to develop gynogenic embryos in each cultivar, demonstrating the influence of individual donor plants. These results are in accordance with Javornik *et al.* (1998) and Bohanec and Jakše (1999), who observed the existence of a strong genetic effect.

Flow cytometry analysis demonstrated that 62.5 % of the total regenerants (sum of the three experiments) were haploid, 25 % were diploid while 12.5 % were mixoploid (haploid and diploid). This fact was already reported by Campion *et al.* (1992) who stated that some regenerants

Table 4. Effect of putrescine inflorescence stalk injection on the gynogenic embryo regeneration rate from flowers of onion cvs. Nav and Val cultivated in basal medium plus 0.002 g dm^{-3} 2,4-D, 0.002 g dm^{-3} BA and 3 g dm^{-3} gellam gum. Different letters in the column indicate significant differences between treatments for each cultivar ($P \leq 0.05$).

Cultivar	Putrescine [g dm^{-3}]	Cultured flowers	Total reg. [%]	Normal plantlet [%]
Nav	0.016	900	0.22 c	0
	0.160	700	0.14 b	1
	1.600	870	0.00 a	0
	control	540	0.00 a	0
Val	0.016	870	0.46 c	4
	0.160	870	0.11 b	0
	1.600	660	0.00 a	0
	control	660	0.45 c	3

have the potential to double spontaneously, while others keep them haploid stage. Furthermore, Nowak (2000) observed that the proportion of haploid/diploid plants obtained were similar to our results and proposed that this phenomenon might be connected with the genetic structure of donor plants. In contrast, Jakše *et al*. (2003) reported a frequency of spontaneous genome doubling of less than 10 %. A higher spontaneous diploidization rate could be an advantage because it reduces the need of using chromosome doubling agents.

In conclusion in our experimental conditions the embryo regeneration and the number of normal plantlets obtained were affected by the concentration of the gelling agent and strongly varied with genotype. Spraying the umbel with CCC previous to the *in vitro* flower culture improved the gynogenic regenerants rate. The effect of the CCC also depended on the genotype.

Although, various factors influence the efficiency of the onion gynogenesis such as flower bud development, culture conditions and the genotype, according to our results, the most determinant is the last one. The use of CCC was effective to make the Argentinean Cob and Val cultivars into medium responsive genotypes. For Nav cultivar it was possible to improve the frequency of regenerants by putrescine injection at a low concentration.

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