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Scientia Horticulturae 87 (2001) 11–20

SCIENTIA HORTICULTURÆ

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Increasing nitrate/ammonium ratio for improvement of garlic micropropagation

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Accepted 12 April 2000

Abstract

The effect of different culture media with different growth regulator and nitrate/ammonium ratios on split garlic shoots was studied to obtain an optimized micropropagation protocol. Three basal media: MS, BDS and BLM (i.e. BDS modified to obtain a nitrate:ammonium ratio of 35 mM:8 mM) combined with two concentrations of NAA (0.5 or 5 μM) and BAP (1 or 10 μM) were evaluated in three Colorado garlic clones: Español Selección Ascasubi (ESA), Español Selección Médanos (ESM) and I 50. In BLM medium with 5 μM NAA and 10 μM BAP the highest multiplication rates were 140, 542 and 743 shoots per split shoot after three subcultures in 140 days for clones ESA, ESM and I 50, respectively. Comparing the mean rates obtained in MS medium with those obtained in BDS medium in the same time, the latter produced 1.5-, 4- and 2.2-fold increases in the mean multiplication rates for ESA, ESM and I 50 clones, respectively; while BLM produced 5-, 3- and 10-fold increases in the mean multiplication rates. Since BDS and BLM media only differ in their NO_3^-:NH_4^+ ratio we can conclude that the differences in the multiplication rates could probably be due to an increase in the NO_3^- level of the BLM medium and so the multiplication ratios for these garlic clones can be greatly improved by the use of higher levels of nitrogen supplied as nitrate (35 mM NO_3^-:8 mM NH_4^+ ratio) with higher concentrations of growth regulators such as 5 μM NAA and 10 μM BAP.

Keywords: *Allium sativum*; Splitting; Growth regulators; Nitrate:ammonium ratio; Micropropagation

Abbreviations: BAP, 6-benzylaminopurine; BDS, Dunstan and Short medium (1977); BLM, BDS with 1 g l^{-1} $Ca(NO_3)_2{\cdot}4H_2O$; ESA, Español Selección Ascasubi; ESM, Español Selección Médanos; MS, Murashige–Skoog medium (1962); NAA, naphthaleneacetic acid; 2iP, N_6-(Δ-isopentenyl)adenine.

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0304-4238/01/$ – see front matter
PII: S0304-4238(00)00165-5

1. Introduction

In micropropagation of *Allium* spp., regeneration and multiplication of plantlets is affected by composition of the culture medium, combination of growth regulators used and the kind of explants (Novak et al., 1986a,b; Novak, 1990).

Regarding the culture medium, Dunstan and Short (1977) improved the growth rate of onion callus by adjusting nutritional and hormonal ratios. They compared macronutrient levels of MS (Murashige and Skoog, 1962) and B5 (Gamborg et al., 1968) media with the levels required for onion growth in field conditions and observed that phosphate and nitrate concentrations in the culture medium probably were suboptimal. They formulated a new medium (BDS) which is a modification of B5 containing $NH_4H_2PO_4$ and NH_4NO_3 added to increase $PO_4H_2^-$, NO_3^- and NH_4^+ levels, thus changing NO_3^-/NH_4^+ ratio from 25 mM/2 mM in B5 medium to 29 mM/8 mM in BDS medium. Novak et al. (1986a,b) proposed a micropropagation protocol of garlic (*Allium sativum* L.) through meristem-tip culture and shoot multiplication using BDS medium supplemented with 1 μM NAA with addition of 1 or 5 μM BAP at multiplication stage. Actually, in the germplasm bank of Gatersleben, Germany, stocks of virus free garlic and onion are conserved and micropropagated in BDS medium (Keller and Lesemann, 1997). In multiplication of garlic shoots, other authors used MS medium supplemented with 0.5 μM NAA and 10 μM cytokinins (kinetin, BAP or 2iP) and obtained a low multiplication ratio (Lee et al., 1988; Lee and Lee, 1994). In fact, with cv. Seosan, Lee and Lee (1994) obtained only two shoots per shoot-tip culture in MS medium with 2.7 μM NAA and 9.2 μM kinetin, after 4 months. Nagakubo et al. (1993) using LS medium (Linsmaier and Skoog, 1965) with 5 μM NAA and 10 μM BAP and a KNO_3:NH_4Cl ratio adjusted to 56.5 mM NO_3^-:3.5 mM NH_4^+, obtained 128 shoots per shoot in cv. Howaito roppen after three subcultures during 8 months.

The objective of this work was to determine the effect of the use of different basal culture media (MS, BDS and BLM) with different NO_3^-:NH_4^+ ratios in combination with different growth regulator concentrations on the multiplication ratio of three Colorado garlic clones.

2. Materials and methods

Three Colorado garlic clones provided by the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina, were used: Español Selección Ascasubi (ESA), Español Selección Médanos (ESM) and I 50. These clones were established and micropropagated through a well-known protocol by Moriconi et al. (1990). In brief, meristem-tip culture was initiated in MS medium supplemented with

Table 1
Nitrogen source and NO_3^-/NH_4^+ ratio of the three culture media (MS, BDS and BLM) and multiplication ratio of the three Colorado garlic clones (ESA, ESM and I 50) in the third subculture

Nitrogen source	NO_3^-/NH_4^+ ratio		
	MS	BDS	BLM
KNO_3	19/0	25/0	25/0
NH_4NO_3	21/21	4/4	4/4
$NH_4H_2PO_4$	–	0/2	0/2
$(NH_4)_2SO_4$	–	0/2	0/2
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	6/0
Total NO_3^-:NH_4^+ ratio	40/21	29/8	35/8
Ratio	1.9	3.6	4.4
Clones	Mean multiplication ratio (S.D.)[a]		
ESA	2.95 (2.75) b	3.24 (3.68) b	5.13 (4.36) a
ESM	3.43 (3.92) b	4.63 (4.31) b	8.48 (9.80) a
I 50	2.99 (2.31) b	4.05 (3.03) b	6.69 (5.63) a

[a] Letters in each row show $P \leq 0.05$ by Tuckey–Kramer test.

0.57 μM IAA and 0.46 μM kinetin, 30 g l^{-1} sucrose, 7 g l^{-1} agar, pH 5.7. Meristems regenerated into plantlets within 2 months. Then, two subcultures were done in a MS multiplication medium supplemented with 1.61 μM NAA, 14.7 μM 2iP, 30 g l^{-1} sucrose, 7 g l^{-1} agar, pH 5.7 (Moriconi et al., 1990) under 16 h photoperiod (30 μmol s^{-1} m^{-2}) at 25±2°C for 3 months.

Plant material arising from previous micropropagation protocol was used to test three basal media: MS (Murashige and Skoog, 1962), BDS (Dunstan and Short, 1977) and BLM. These culture media differ in their nitrogen source and their NO_3^-:NH_4^+ ratios (Table 1). BLM is a modification of BDS medium prepared by adding 1 g l^{-1} $Ca(NO_3)_2{\cdot}4H_2O$ to obtain a 35 mM NO_3^-:8 mM NH_4^+ ratio without modifying the NH_4^+ concentration of the original medium. These three basal media were supplemented with two concentrations of NAA (0.5 or 5 μM) and BAP (1 or 10 μM) in a factorial design. Shoots were selected by their diameter at the stem base, and were longitudinally cut to obtain split shoots as explants. Survival rate was registered as the number of explants alive compared to total explants. The multiplication ratio was expressed as the number of shoots per split shoot obtained at the end of each subculture. Three subcultures were done at 45-day intervals under the same growing conditions as described above. Treatments were assigned at random and sample treatments were unweighted. This study started with 12–22 explants per treatment depending on the availability of each clone. Data were subjected to two-way ANOVA test and comparison of media was done using the Tuckey–Kramer test.

3. Results and discussion

Due to either death or inability to regenerate new shoots, survival rates for all the three clones were between 70 and 100%. Hulscher et al. (1992) observed that this fact may be attributed mainly to the physiological response of explants to subculture.

Calli were observed in different proportions (0–100%) depending on clones, basal media and concentrations of growth regulators. The highest proportion of explants with callus was observed at the highest concentrations of NAA and BAP in the second subculture. The lowest callus formation was observed in BLM medium during the third subculture, with the exception of the treatment with 5 μM NAA and 1 μM BAP on clones ESM and I 50. These calli yielded vitrified shoots that were easily identified, separated and discarded and the remaining explant was then subcultured. It is important to note that callus development appeared at the basal plate with no effect on shoot formation at the meristematic axillary zone.

Between 0 and 45% of the explants in all garlic clones showed bulb formation, which is apparently not related to the number of subcultures. A relationship between bulbification ratio and treatments was observed; for all basal media, the lowest bulb production was closely related with the highest concentrations of regulators, noticeably with 10 μM BAP. Increased bulb formation was obtained with BDS and BLM supplemented with 0.5 μM NAA and 1 μM BAP and with MS supplemented with 5 μM NAA and 1 μM BAP (Fig. 1).

Differences in the multiplication ratio were obtained between clones, between subcultures in each clone and between different concentrations of growth regulators and composition of the basal medium (Table 2). The highest multiplication ratio for all clones occurred in BLM medium, generally showing increased values with the highest NAA and BAP concentrations and with subsequent subcultures (Fig. 2). Whatever the basal culture medium used, treatments supplemented with 5 μM NAA and 10 μM BAP produced the highest multiplication ratio ($P \leq 0.01$) in ESA and ESM clones. For the I 50 clone, the best multiplication ratio occurred in BLM medium with 0.5 or 5 μM NAA and 10 μM BAP (Fig. 2).

For the ESA clone, the highest multiplication ratios were 4, 5 and 8 shoots per split shoot in MS, BDS and BLM medium, respectively; these values were obtained in the treatments with 5 μM NAA and 10 μM BAP. In the ESM clone the multiplication ratio decreased with successive subcultures in MS medium with the exception of the treatment with 5 μM NAA and 10 μM BAP which produced seven shoots per split shoot at the end of the third subculture. However, using the same concentrations of growth regulators, BDS medium produced nine shoots per split shoot in the second subculture and BLM medium produced 14 shoots in the third subculture. Multiplication ratio in ESM increased with successive

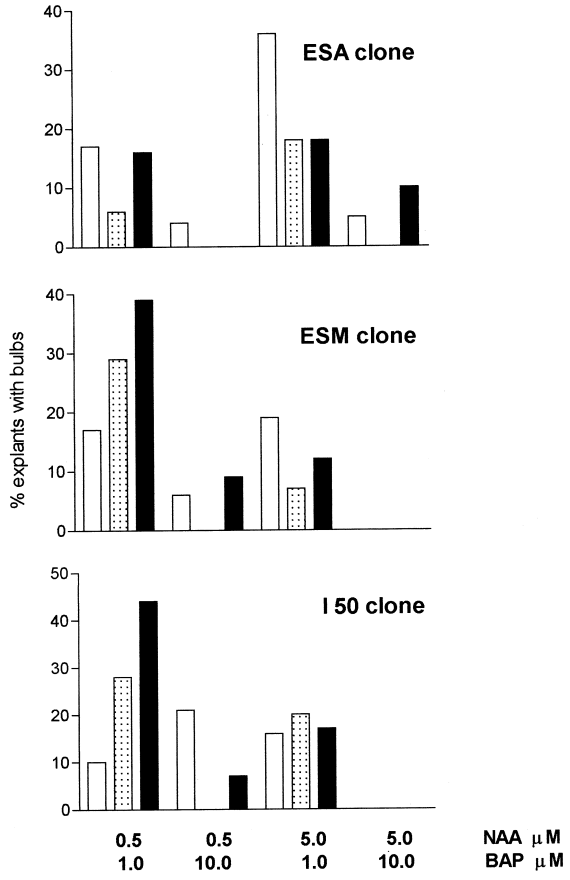

Fig. 1. Percentage of explants with bulb formation of the three Colorado garlic clones (ESA, ESM and I 50) in three basal media (see Section 2): (▭) MS; (▩) BDS and (■) BLM supplemented with 0.5 or 5 μM NAA and 1 or 10 μM BAP at the end of the third subculture.

subcultures in BLM medium, particularly in the presence of 10 μM BAP. In the case of the I 50 clone cultured on either MS or BDS media, the multiplication ratio slightly decreased towards the third subculture. For this clone, BLM medium with 5 μM NAA and 10 μM BAP produced the highest multiplication ratios: eight shoots per split shoot in the first and second subcultures and 11 shoots in the third subculture (Fig. 2). Fig. 3 shows representative samples of in vitro multiplication response in I 50 clone at the end of the second subculture.

According to a previous micropropagation protocol (Moriconi et al., 1990), the values of the propagation rate in MS multiplication medium were 20, 17 and 15 shoots per shoot after establishment and two subcultures in 150 days for ESA, ESM and I 50 clones, respectively (data not shown). These rates are similar to the 15 shoots per shoot obtained in 115 days with a Colorado garlic clone in the same

Table 2
Two-way ANOVA for comparison of basal media and growth regulator treatments for each garlic clone (ESA, ESM and I 50) during three subcultures

	Clones								
	ESA			ESM			I 50		
	Subculture 1	Subculture 2	Subculture 3	Subculture 1	Subculture 2	Subculture 3	Subculture 1	Subculture 2	Subculture 3
Variability									
Media (M)	NS[a]	*	***	NS	***	***	NS	***	***
Growth regulators (GR)	NS	NS	**	NS	***	***	**	**	***
M×GR	NS	NS	NS	NS	NS	NS	NS	NS	*

[a] Non significant.
* Significant at $P \leq 0.05$.
** Significant at $P \leq 0.01$.
*** Significant at $P \leq 0.001$.

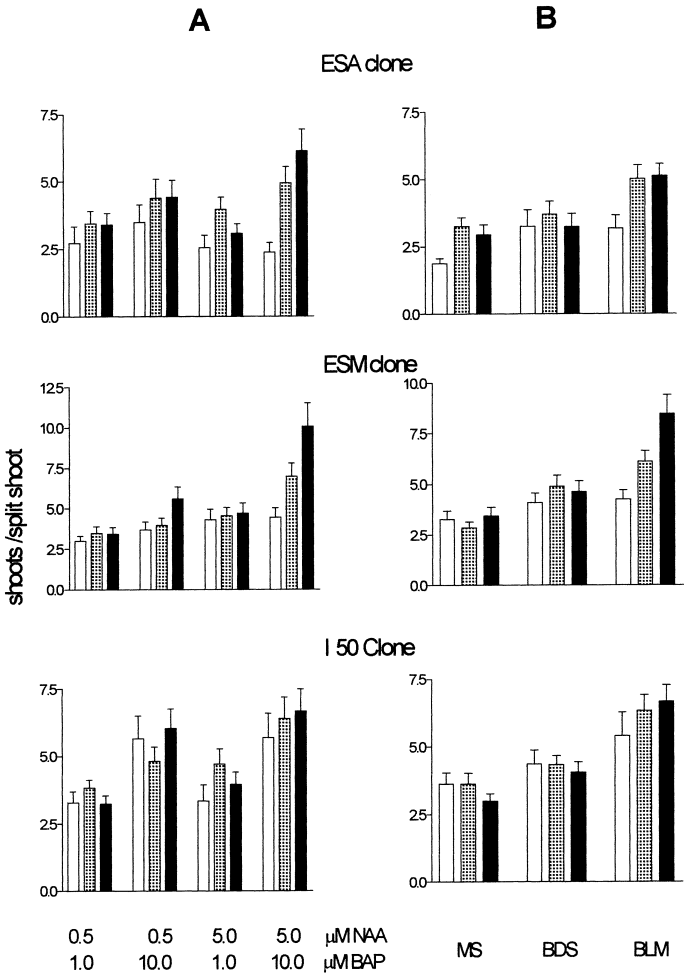

Fig. 2. In vitro multiplication ratio of the three Colorado garlic clones (ESA, ESM and I 50) expressed as the mean number of shoots per split shoot every 45 days at the end of the first (▭), second (▩) and third (■) subculture: (A) mean number of shoots per split shoot as a function of growth regulators, i.e. 0.5 and 5 µM NAA combined with 1 and 10 µM BAP; (B) mean number of shoots per split shoot as a function of the culture media: MS, BDS and BLM. Bars represent mean values with standard deviations.

medium (Moriconi et al., 1990). In this medium, significant differences were not found in propagation between clones. However, composition of basal media and growth regulator concentrations in BDS and BLM did markedly affect the multiplication response between clones. These differences indicate the occurrence of one factor limiting the growth of the explant in the composition of the MS medium.

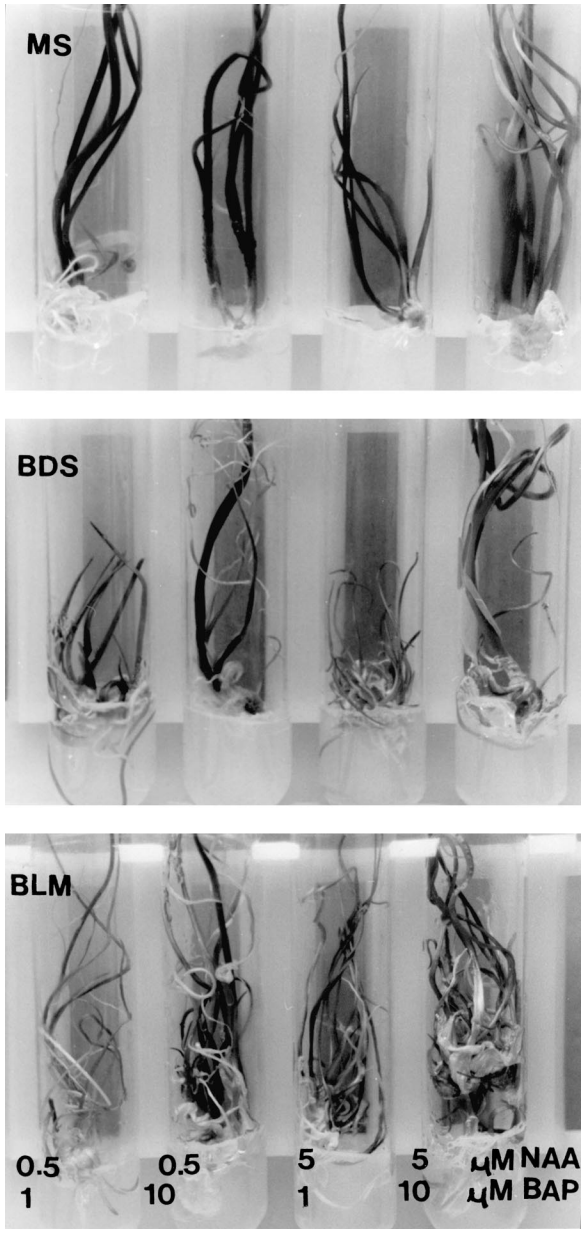

Fig. 3. Representative samples of in vitro multiplication of I 50 garlic clone at the end of the second subculture in the three basal media (see Section 2), MS, BDS and BLM, supplemented with 0.5 or 5 μM NAA and 1 or 10 μM BAP.

Comparing the mean rates obtained in MS medium (27, 78 and 77 shoots per split shoot) with those obtained in BDS medium at the same time, the latter produced 1.5-, 4- and 2.2-fold increases (48, 306 and 157 shoots per split shoot) in the mean multiplication rates for ESA, ESM and I 50 clones, respectively. While for the same clones, BLM produced 5-, 3- and 10-fold increases in the mean multiplication rates (i.e. 140, 542 and 743 shoots per split shoot). Since BDS and BLM media only differ in their NO_3^-:NH_4^+ ratio we can conclude that the differences in the multiplication rates could probably be due to an increase in the NO_3^- level of the BLM medium (Table 1).

Considering pre-existing protocols for garlic micropropagation, the present work shows that in vitro multiplication rates for these garlic clones can be greatly improved by the use of higher levels of nitrogen supplied as nitrate (35 mM NO_3^-:8 mM NH_4^+ ratio) at higher concentrations of growth regulators such as 5 μM NAA and 10 μM BAP.

Acknowledgements

We thank Ing. Silvia Delmastro for helpful technical assistance. This work was supported by grants from Universidad Nacional del Sur and CERZOS, CONICET, Argentina.

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