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Establishment of efficient method for callus culture and shoot regeneration of local Indian garlic (var. Yamuna safed)

Rashmi Kapoor[1], Sekh Abdul Nasim[2], Mahmooduzzafar[3] and Abdul Mujib[4]

[1,2,3] Environmental Biotechnology Laboratory, Department of Botany, Hamdard University, New Delhi-110062, India
[4] Plant Tissue Culture Laboratory, Department of Botany, Hamdard University, New Delhi-110062, India

Abstract
The experiment was designed to investigate the regeneration potentiality of a garlic variety (Yamuna Safed (G1)), and also to develop an efficient protocol for regeneration of garlic via callus culture. Higher percentage of callus was initiated from the combination of BAP and 2, 4-D at 2.0 mg/l and 0.25 mg/l respectively. The hormone 2, 4-D, commonly used in tissue culture in garlic was found to be detrimental when used at the levels described in literatures. Rooting of individual shoots was induced after transfer to medium without growth regulator. The plantlets were established in the soil after acclimatization.

Keywords: *In vitro,* Garlic, Callus

INTRODUCTION

Garlic (*Allium sativum* L.) is an important and widely cultivated plant used for food and medicinal purposes. It is propagated exclusively vegetatively, a process that has a low coefficient of multiplication and potential for transmission of viral diseases [1]. The propagation rate of garlic in the field is very slow and it takes many years to produce a new variety for practical cultivation. In spite of many efforts to develop *in vitro* techniques for garlic micro propagation [1], an efficient system for fast production of callus and subsequent regeneration is still lacking for this crop.

The propagation of garlic using *in vitro* technique has been studied by numerous workers [2, 3, 4]. Although various explants such as shoot tip [2, 5, 6], stem disc [7, 8] and root [9, 10] were successfully used to induce callus, rate of shoot regeneration from callus is still low. Recently, improvement of shoot regeneration by adding various combinations of plant growth regulators to media have been reported [4, 7] and encouraging results are shown that are helpful in obtaining a large quantity of regenerated shoots for commercial use. However, systematic experiments on both callus and the shoot proliferation are still not enough, which may overlook the potential combinations of certain plant growth regulators that are more suitable for shoot multiplication. Moreover, the preliminary analysis of nutritional constituents of different garlic varieties at RRS Karnal, India (2001-2002) reveals the variety under study (Yamuna Safed) to be more nutritionally endowed. An attempt was thus undertaken with the following objectives:

- Development and identification of the best protocols for callus production and regeneration of garlic.
- Study the potentiality of callus and shoot induction ability of a local Indian garlic variety.

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*Corresponding Author
Rashmi Kapoor

Environmental Biotechnology Laboratory, Department of Botany, Hamdard University, New Delhi-110062, India

Tel: +91-9899101335; Fax: +91-1126059663
Email: kapoorashmi@gmail.com

MATERIALS AND METHODS

Plant material

Local Indian garlic (*Allium sativum* L. cv. Yamuna safed), obtained from National Horticultural Research and Development Foundation, Nasik (Maharashtra) was used for the present study. Plant identity was confirmed and voucher specimen (IC-375117) was deposited at National Bureau of Plant Genetic Resources, New Delhi, India.

Cloves of garlic were used as explant. Bulbs, harvested from 4–5 months old plants, were stored at 15 °C and kept for 3 weeks at 5 °C in order to break dormancy before the experiments.

Explants Preparation

Healthy garlic cloves were selected and washed under running water for 30 min, surface sterilized with 0.1% $HgCl_2$ for 10 min and 70% ethanol for 30 seconds and washed several times with sterilized distilled water. Garlic cloves were divided into basal, middle and tip part; and inoculated on Murashige and Skoog medium [11]. Nearly 3-5 mm cloves were transferred to culture tubes containing 15 ml of solidified basal medium supplemented with various concentrations of BAP, NAA, 2, 4-D, IAA, Kn and 2iP.

Medium and culture conditions

Murashige and Skoog (1962) basal nutrient medium was used to carry out the present investigation. Both natural and synthetic plant growth regulators were used. Besides the growth regulators, some other chemicals like carbohydrates, sugar, alcohol, gelrite (a solidifying agent) were also added in the MS medium which influence the growth and developmental processes. Their optimum concentrations were established by trial and error method. All medium components were added and adjusted to pH 5.6 prior to autoclaving at 121 °C, 104 kPa for 20 min. All the cultures were incubated at 25 ± 2 °C in a culture room under 16-h photoperiod

provided by cool white fluorescent light giving intensity of 40 Wm^{-2} for seven weeks. Most of the chemicals used for the preparation of media were of analytical grades. Stock solutions were prepared and stored as per need.

RESULTS AND DISCUSSION

Focus of this study was to establish an efficient method for callus culture and shoot regeneration (via unorganized calli) of garlic.

It was observed that (table1A, expt X_3) callus production was greatest on the medium containing BAP (2 mg/l), and low concentration of 2, 4-D (0.25 mg/l).

Table 1A. Effect of callus formation media and regeneration media on percentage of explants producing callus, percentage of organogenic explants, and shoots produced per gram of callus in *A. sativum* L.

	Medium	Plant growth regulators, mg/l				Callus formation %	Regeneration %	No. of shoots/gm
		Callus formation medium						
		BAP	2, 4-D	NAA	2iP			
X_1	1	0.00	0.50	0.00	0.00	49.94 ± 5.34^{b}	15.33 ± 1.77^{b}	0.61 ± 0.21^{b}
X_2	2	2.00	0.50	0.00	0.00	29.32 ± 4.41^{c}	38.41 ± 2.99^{a}	2.50 ± 0.77^{b}
X_3	3	2.00	0.25	0.00	0.00	83.71 ± 5.12^{a}	35.31 ± 2.25^{ab}	14.42 ± 1.99^{a}
Y_1	4	2.00	0.00	1.00	0.00	77.81 ± 3.98^{a}	47.88 ± 3.88^{a}	9.73 ± 2.11^{ab}
Y_2	5	2.00	0.00	2.00	0.00	75.31 ± 4.98^{a}	52.11 ± 2.93^{a}	8.77 ± 1.98^{ab}
Y_3	6	2.00	0.00	2.50	0.00	71.11 ± 3.12^{a}	58.81 ± 2.65^{a}	7.34 ± 2.11^{ab}
Y_4	7	2.00	0.00	3.00	0.00	69.52 ± 4.11^{a}	50.11 ± 3.01^{a}	6.80 ± 1.73^{ab}
Y_5	8	2.00	0.00	4.00	0.00	64.50 ± 2.87^{a}	42.11 ± 3.76^{a}	5.69 ± 1.97^{ab}
ANOVA								
F	-	-	-	-	-	12.77***	3.77*	1.98*
P	-	-	-	-	-	0.007	0.002	0.00

Each value represents mean ± SEM from three independent experiments having three replicates. Different letters in a column are significantly different at p = 0.05 level.

Callus production was found to be least on the medium containing BAP (2 mg/l) and higher concentration of 2, 4–D (0.5 mg/l) without NAA (table 1A, expt X_2) indicating that a slight increase in 2,4-D content resulted in a significant decrease in callus formation. However, researchers have observed 2, 4-D as the best auxin for callus induction in monocots and even in dicots [12]**.** High concentrations of 2, 4-D (2.0 -5.0 mg/l) have also been reported to be favorable for high callus formation from tuber explants in potato cultivars [13]. The use of similar high concentrations of 2, 4-D (3 mg/l) have been advocated [14] to be most effective concentration for callus induction from internodes and leaf explants in various potato cultivars used.

Regeneration, however, was found to be good on both of these media. And it was evident from expt Y_1 that NAA stimulate shoot formation. However, 2, 4-D has been reported to be more effective than NAA for callusing [15]. Though it was observed from expt Y (table1A) that there was no significant difference among NAA concentrations in their effects on callus formation or regeneration but shoot formation declined with increasing concentration of NAA (table 1A). Callus formation and regeneration for all five treatments in expt Y (Y_1-Y_5) were not significantly different from those for the best treatment in expt X (X_3). Shoot formation in the case of both best treatments (expt X and expt Y) did not differ significantly. Similarly, there was no significant difference in case of percentage of regeneration or number of shoots per gram among the media used in all treatments in expt Z. (Table 1B).

It was evident from the experiment Y (table 1A) that high doses of NAA promote regeneration but reduce callus formation. By comparing callus formation media without 2, 4-D (expt Y) with medium in expt X_3 which contains 0.25 mg/l 2, 4-D, we conclude that low levels of 2, 4-D increase the percentage of explants producing callus and the number of regenerated shoots per gram of callus.

From our study, it was worth noting that 2, 4-D is not essential for callus induction and regeneration and can be unfavorable when used in concentrations similar to those described in earlier published protocols [1, 7]. The combination of high concentration of NAA and BAP produced excellent results in terms of percentage of callus formation and shoot regeneration and in some case, in levels of shoots per gram.

Each value represents mean ± SEM from three independent experiments having three replicates.

Our studies lead us to point out from data obtained from tables (table 1A & 1B) that regeneration medium did not show any significant differences in all the tested treatments (table 1B). The callus formation medium differed significantly both in terms of percentage of regenerating explants and number of shoots per gram (table 1A). While organogenic media did not explain the importance of the medium used to initiate callus in the tissue culture process. The study after analyzing all the data procured suggested that the best hormonal combination for callus induction is the one used in expt X_3 which is a combination of 2 mg/l BAP and 0.25 mg/l 2,4-D. The medium used in expt Y_3 which is a combination of 2 mg/l BAP and 2.5 mg/l NAA was found to be the best medium for shoot regeneration.

Table 1B. Effect of regeneration medium on callus production, percentage of organogenic explants, and shoots produced per gram of callus in *A. sativum* L.

Expt	Medium	Plant growth regulators, mg/l				Callus formation %	Regeneration %	No. of shoots/gm
		BAP	K_n	NAA	IAA			
Z_1	1	0.00	0.00	0.00	0.00	----	61.22 ± 4.11	4.98 ± 1.22
Z_2	2	2.00	0.00	0.00	0.00	----	63.84 ± 4.99	7.75 ± 3.10
Z_3	3	0.00	4.00	1.00	0.00	----	57.33 ± 3.21	6.99 ± 2.48
Z_4	4	0.00	0.00	0.00	0.01	----	55.43 ± 3.22	5.91 ± 2.00
ANOVA								
F	-	-	-	-	-	-----	0.77^{ns}	0.83^{ns}
P	-	-	-	-	-	-----	0.000	0.010

Each value represents mean ± SEM from three independent experiments having three replicates.

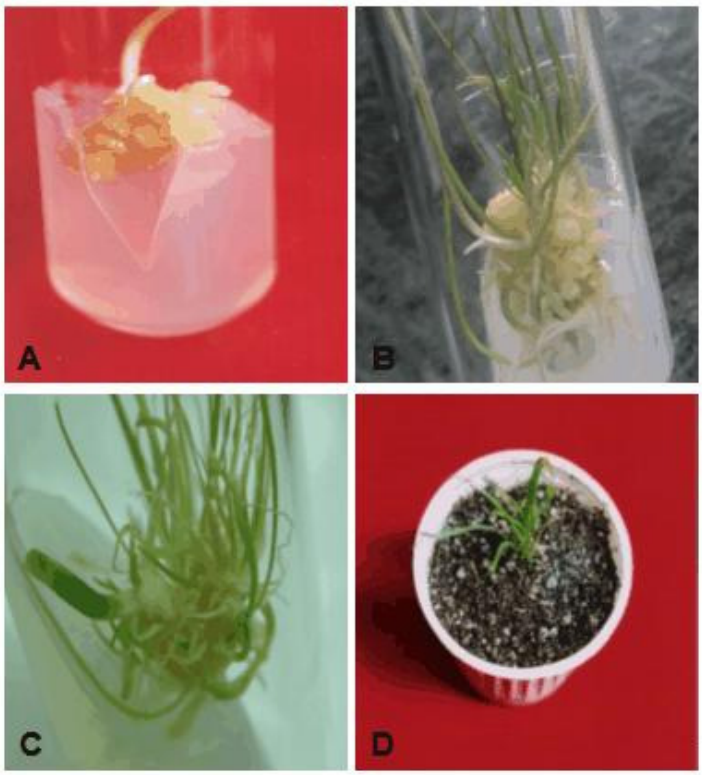

Fig 1. *In vitro* plant regeneration in *Allium sativum* L. (A) Callus formation in media supplemented with 2 mg/L BAP and 0.25 mg/L 2,4-D. (B) Shoot regeneration in media supplemented with 2 mg/L BAP and 2.5 mg/L NAA. (C) & (D) Complete plantlet before transfer to field.

CONCLUSION

The local Indian garlic variety (Yamuna Safed) is nutritionally more valuable as suggested by preliminary reports. The protocol we report here is a reliable alternative method for propagation of this garlic variety. Our study suggests that a combination of 2 mg/l BAP and 0.25 mg/l 2,4-D is optimum for callus induction while 2 mg/l BAP and 2.5 mg/l NAA is the best medium combination for shoot regeneration in this garlic variety. The improvement in regeneration rate will also help develop systems to increase variations in this sterile plant.

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