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RAPID PRODUCTION OF VIRUS-FREE PLANTLETS BY SHOOT TIP CULTURE *IN VITRO* OF PURPLE-COLOURED SWEET POTATO (*IPOMOEA BATATAS* (L.) LAM.)

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Abstract

A rapid virus-free seedlings formation protocol was established for purple-coloured sweet potato by shoot tip culture *In vitro*. The effects of two factors, namely BAP, NAA and their interaction, on callus, roots, buds and rooted plantlets initiation were evaluated by orthogonal design with two factors and four levels. The variance analysis of the experimental results showed that the actions of the two factors and their interaction had significantly different effects on callus, bud, root and rooted plantlet initiation. The best medium for adventitious bud induction was the combination of solid MS supplemented with 1.0 mg l^{-1}BAP. However, the best medium for rooted plantlet was the combination of solid MS supplemented with 0.5 mg l^{-1}BAP and 0.2 mg l^{-1}NAA. Rooted plantlets were acclimatized to greenhouse conditions and appeared normal. The plantlets from shoot tip tissue culture were transplanted successfully. At the same time, the regenerated seedlings were surveyed by the method of indicator plant and enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA), and the virus-free plantlets of purple-coloured sweet potato was obtained. *In vitro* shoot tip culture can be a useful tool in the provision and conservation of virus-free plantlets of purple-coloured sweet potato.

Introduction

Ipomoea batatas (L.) Lam., popularly known as sweet potato, is a native American plant belonging to the family Convolvulaceae, order Polemoniales (Oggema *et al*., 2007) and is primarily produced in China, which accounts for 80.9% of the world production, but also in Africa and the Americas (Santa-Maria *et al*., 2009). Purple-coloured sweet potatoes developed in Japan in 1990s (Yang *et al*., 2006) and several reports have indicated that the anthocyanins in purple-coloured sweet potatoes displayed antioxidative or radical-scavenging activity and exerted several health-promoting functions in humans (Konczak-Islam *et al*., 2003; Suda *et al*., 2003; Rabah *et al*., 2004).

Because of the more frequent introduction and exchange of sweet potato cultivars and clonal propagation, many sweet potato are generally infected with viruses such as sweet potato feather mottled virus (SPFMV), sweet potato mild mottled virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), sweet potato latent virus (SPLV) and damage from virus diseases has become increasingly serious. Sweet potato virus diseases cause declining yield, quality and resistance to insects. However, with the rapid expansion of the natural anthocyanin pigments industry in China, the need for purple-fleshed sweet potato is dramatically increasing.

Biotechnological approaches are of potential importance to sweet potato improvement. Plant tissue culture and regeneration techniques are useful in the production of somaclonal variants and the development of transgenic plants and are valuable tools in the understanding of basic plant biology. Since the invent of *In vitro* techniques, a lot of interest

has been generated in the recent year for the rapid multiplication of virus free plant through apical meristem (Naz *et al.,* 2009). There are some reports of shoot tip culture of sweet potato (Kuo *et al*., 1985; Kong *et al*., 1998), but little information was available on the reports of shoot tip culture of purple-coloured sweet potato. The present paper given are without as to how to use a designed orthogonal test to successfully investigate the influence of some factors such as plant growth regulators including BAP and NAA and their interaction on rapid seedlings formation *In vitro* shoot tip culture of purple-coloured sweet potato.

Material and Methods

Plant material and explant preparation: Nodal segments (3-5 cm) with 1-2 armpit buds were taken from the apical portions of purple-fleshed sweet potato cv."Zishu No.10", the leaf stalks and leaves were sliced off. The explants were washed with detergent under running tap water for 30 minutes, nodal segments were dipped into 70% alcohol for 30secs, followed by 0.1% $HgCl_2$ and Tween-80 (one or two drops per 100ml) for 8-9 min. The explants were finally rinsed 4-5 times with sterilized distilled water. Surface-sterilized explants were germinated and incubated on MS (Murashige & Skoog, 1962) solid medium for 30d in a growth cabinet with a 12-h photoperiod under cool-white light (45-55 μmol m^{-2} s^{-1}) at 28±0.5◦C. All media were adjusted to pH 5.8, and 0.8% (w/v) agar and 30 g l^{-1} sucrose were added prior to autoclaving at 121◦C for 20 min (103 kPa).

Shoot tips of purple-coloured sweet potato 0.2-0.5 mm in length were cut from the sterile seedlings under light microscope in super-clean workbench and incubated onto MS solid medium supplemented with varying levels of BAP alone or in combination with NAA (Table 1), and the test tubes containing shoot tip were then placed at the same culture conditions mentioned above. The number of calli, buds and roots was recorded and the frequency of callus formation, root differentiation and shoot regeneration were calculated after 45d of culture from the initial inoculation time.

Two factors including BA and NAA, both selected at four different concentration levels were studied. The orthogonal table of two factors and four levels was selected for studying the two factors and the interaction (BAP×NAA), effects on initiating induction of callus, roots, buds and rooted shoots (Table 1). Sixteen treatments were arranged according to the design and the whole experiment was repeated three times.

Virus detection

The method of indicator plants detection: *Ipomoea setosa* Ker is a convenient indicator plant to detect sweet potato viruses. Eighteen seedlings of regenerated plants of purple-coloured sweet potato were randomly selected. Plantlet was cut in two and the lower section are used as the scion and grafted on a plant of *I. setosa*.

The method of ELISA: Eighteen seedlings of regenerated plants of purple-coloured sweet potato were randomly selected. Sweet potato feather mottled virus (SPFMV), sweet potato mild mottled virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), sweet potato latent virus (SPLV), C-6 and C-8 were detected by enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA) according to the prescribed method of product purchased from Biotechnology Center, Academy of Agricultural Sciences of Shandong, China. The colour reaction produced by tested samples were compared with known negative control.

Table 1. Effects of plant growth regulators on shoot regeneration from shoot tip explants of purple-coloured sweet potato after 45d.

BAP (mg l^{-1})	NAA (mg l^{-1})	% of explants producing callus	% of explants producing shoots	% of producing rooted shoots explants
0	0	0.00	0.00	0.00
	0.2	0.00	0.00	0.00
	0.5	10.23 ± 0.60f	0.00	0.00
	0.8	36.43 ± 0.72e	9.13 ± 0.90d	9.13 ± 0.90b
0.5	0	40.03 ± 1.56d	0.00	0.00
	0.2	100.00 ± 0.00a	11.47 ± 0.49c	11.47 ± 0.49a
	0.5	100.00 ± 0.00a	2.93 ± 0.56f	2.93 ± 0.56d
	0.8	100.00 ± 0.00a	0.00	0.00
1.0	0	97.03 ± 0.42b	20.83 ± 1.20a	4.47 ± 0.30cd
	0.2	92.57 ± 0.42c	0.00	0.00
	0.5	100.00 ± 0.00a	5.80 ± 0.91e	5.80 ± 0.91c
	0.8	100.00 ± 0.00a	0.00	0.00
1.5	0	91.33 ± 1.92c	16.67 ± 0.42b	4.17 ± 0.56cd
	0.2	92.23 ± 0.64c	0.00	0.00
	0.5	100.00 ± 0.00a	0.00	0.00
	0.8	100.00 ± 0.00a	0.00	0.00

Values represent means ± SE. Values followed by the same letter are not significantly different at $p<0.05$ according to Tukey's least-significant-difference test range test.

Statistical analysis: All experiments were repeated three times. Statistical significance of the results was determined using the least-significant-difference test (Tukey, 1953).

Results

Effects of plant growth regulators on shoot regeneration: Three days after inoculation, callus begin to form, 15d after inoculation, new buds (Fig. 1A) and roots appeared, the number of emerging calli, buds and roots was counted after 45d and related results calculated are summarized in Table 1.

Analysis of variance (ANOVA): In order to evaluate the significance of the main effects of hormones (BAP and NAA) and their interaction (BAP×NAA) and to identify the optimum medium, ANOVA (analysis of variance) based on the data presented was carried out and the results are presented in Table 2, 3, 4, 5. The two factors, namely BAP, NAA and all interactions between them had significantly different effects on callus, bud, root and rooted shoot initiation ($p<0.05$). BAP had the greatest effect on callus initiation, followed by NAA and the combination of BAP and NAA; the combination of BAP and NAA had the greatest effect on bud initiation, followed by NAA and BAP; NAA had the greatest effect on root initiation, followed by the combination of BAP and NAA and BAP; the combination of BAP and NAA had the greatest effect on rooted shoot induction, followed by BAP and NAA.

The best medium for bud induction was the combination of solid MS supplemented with BAP (1.0 mg l^{-1}). The best medium for rooted shoot was the combination of solid MS supplemented with 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} NAA .

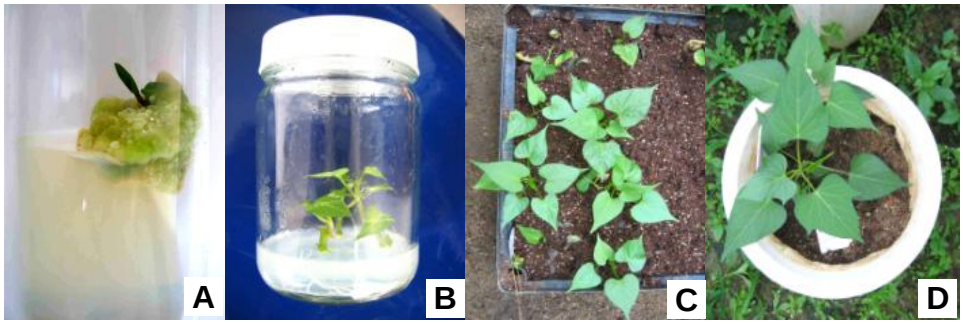

Fig. 1. Plant regeneration from shoot tip explants of purple-coloured sweet potato.
A. Callus formation from shoot tip explant and bud appeared on the surface of callus after 15d; B. Rapid propagation of plantlet of purple-coloured sweet potato (MS without hormone); C. Micropropagated plant in containers with sterile soil after 45d; D. Micropropagated plant in pots containing a mixture of soil vermiculite and sand in equal proportions after 53d.

Transplantation of the plantlet: When grown on the above selected best medium, a single bud could multiply 16 times in a month period, which meant that a single bud could multiply theoretically about 16^{12} times per year under proper conditions (Fig. 1B). On optimal medium, purple-fleshed sweet potatoes grow very well, rooted readily on MS with low concentration NAA or without hormone. The rooted plantlets were transferred to containers with sterile soil (Fig. 1C) and maintained under high humidity and acclimatized in a mist house for two weeks. Humidity was reduced gradually, and the plants were transferred to pots containing a mixture of soil vermiculite and sand in equal proportions (Fig. 1D). There was no mortality in the transplanted plants, and all plantlets appeared healthy and normal. Young purple-fleshed sweet potatoes were planted in the field very successfully.

The results of viruses detection: After about 15d, obvious symptoms, such as mosaic, curled or wrinkled leaves, vein clearing, were observed in the leaves on three plants of *I. setosa* grafted in the eighteen seedlings of sweet potato, it suggests that its corresponding cuttings of sweet potato are infected by virus and the three seedlings were thrown away immediately.

At the same time, the result of NCM-ELISA show that four samples among all the eighteen samples indicated positive reaction, which meant that the four samples failed in virus elimination and were thrown away.

Discussion

As far as plant regeneration is concerned, the sweet potato is recalcitrant. There are several reports of *In vitro* organogenesis in sweet potato using explants derived from various organs and tissues, such as stems, petioles, leaves and roots, the frequency of regeneration has been very low and callus cultures do not produce any shoots (Henderson *et al*., 1983; Kuo, 1991; Sihachakr *et al*., 1997). However, the present study indicated that shoot tip cultures not only directly produce callus, adventitious bud and root, but also produce rooted shoots.

Table 2. Statistical analysis of different factors used in the optimization study for the production of the callus induction of purple-coloured sweet potato.

Sources of variance	Degree of freedom	Sum of square	Mean square	F value	F 0.05
NAA	3	4790.28	1596.76	6266.92*	2.92
BAP	3	60291.99	20097.33	78877.50*	2.92
NAA × BAP	9	6278.52	697.61	2737.98*	2.21
error	30	7.64	0.25		

Table 3. Statistical analysis of different factors used in the optimization study for the production of the bud induction of purple-coloured sweet potato.

Sources of variance	Degree of freedom	Sum of square	Mean square	F value	F 0.05
NAA	3	435.56	145.19	321.44*	2.92
BAP	3	120.91	40.30	89.23*	2.92
NAA × BAP	9	1512.88	168.10	372.16*	2.21
error	30	13.55	0.45		

Table 4. Statistical analysis of different factors used in the optimization study for the production of the root induction of purple-coloured sweet potato.

Sources of variance	Degree of freedom	Sum of square	Mean square	F value	F 0.05
NAA	3	3445.23	1148.41	2183.58*	2.92
BAP	3	430.17	143.39	272.64*	2.92
NAA × BAP	9	1758.43	195.38	371.50*	2.21
error	30	15.78	0.53		

Table 5. Statistical analysis of different factors used in the optimization study for the production of the rooted plantlet of purple-coloured sweet potato.

Sources of variance	Degree of freedom	Sum of square	Mean square	F value	F 0.05
NAA	3	4.01	1.34	3.25*	2.92
BAP	3	39.881	13.29	32.36*	2.92
NAA × BAP	9	569.211	63.25	153.94*	2.21
error	30	12.33	0.41		

BAP is considered to be one of the most useful cytokinins for achieving the multiplication and microprogation of the plants (Stfaan *et al*., 1994;). Yucesan *et al*., (2007) reported the experiments using Witloof cichory as materials and involving combinations of two auxins and two cytokinins, the results showed that auxins, when used alone, had no effect on shoot induction since either NAA or IAA produced no shoots at all. Both types of cytokinin, when used alone, were able to produce shoots and BAP was more effective. Ali *et al*., (2008)found that the best shoot induction response of the genotype of sugarcane (CP 77,400) was obtained on MS medium supplemented with 1.0 mg l^{-1} BAP. These findings were consistent with the results of our experiments. Most regeneration was observed only on explants with BAP (Table 1). Maximum regeneration of shoots (20.83%) was observed when 1.0 mg l^{-1} BAP was included in MS medium. Kong *et al*., (2003) reported that MS medium supplemented with 0.1-0.5 mg l^{-1} NAA

was beneficial to plantlets regeneration from subcultured tip meristem in sweet potato. When concentration of NAA was high (to 1.0 mg l^{-1}), tip meristem explant of sweet potato pruduced much callus, plantlet ratio from subcultured tip meristem in sweet potato decreased. These reports were in harmony to the results of the present study. Regeneration efficiencies were a significant genotype-dependent response which observed in industrial 'high starch' sweet potato genotypes by Santa-Maria *et al.,* (2009). In the present study, the percentage of plant regeneration is a low rate (20.83%).This may be related to this particular sweet potato genotype.

The cytokinin-auxin combination has also been used widely for shoot regeneration in various protocols developed for other general cultivars of sweet potato. The present study proved that BAP, NAA and all interactions between them had significant effects on plant regeneration. Maximum rooted plantlet regeneration of shoots (11.47%) was observed when 0.5 mg l^{-1} BA and 0.2 mg l^{-1} NAA were included in MS medium.

The present study also proved that orthogonal design and statistics analysis were very useful for discovering and selecting the best experimental treatment. Once the optimal medium was selected out, rapid multiplication of purple-coloured sweet potato could be established. The successful acclimation of purple-coloured sweet potato plantlets showed that *In vitro* shoot tip culture could be a technique to obtain enough virus-free plantlets for farmers. This could not only solve the shortage of virus-free plantlets, but also be a very practical and useful technique for enhancing the yield of purple-coloured sweet potato.

There are obvious differences between symptomless and diseased sweet potato (Yang *et al*., 2005). The former has the character of fast growth, and dark-green leaves, but the latter grows slowly with small-green leaves or has symptoms such as mosaic on the leaves, vein clearing, chlorosis, curled or wrinkled leaves or less obvious, russet feathery mosaic and ring spots on the old leaves. Although diseased plants can be rejected easily, some latently infected plants infected by SPLV may escape visual selection. So, visual estimation method is not effective enough to reject all infected plants. However, Serology (ELISA) and indicator plant can detect viruses quickly and accurately and so it has become the most common protocol.

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