

Effect of growth regulators on meristem culture and plantlet establishment in sweet potato [*Ipomoea batatas* (L.) Lam.]

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Abstract

Sweet potato shoot apical meristems with 1-2 leaf primordia were aseptically isolated and cultured on liquid or solid Murashige and Skoog (MS) medium supplemented with different plant growth regulators. Primary shoot induction was most effectively promoted by liquid MS medium supplemented with 2.0 mg/l Kn plus 0.5mg/l GA₃. In this combination about 75% of excised meristems responded with an average vigor of 0.79. The shoots were regenerated without intervening any callus formation. The use of TDZ or BAP produced callus instead of shoot development and therefore not recommended for meristem culture. Following primary establishment, the primary shoots were further cultured on semisolid MS medium supplemented with 2.5mg/l Kn+0.5mg/l GA₃ for proper shoot growth. The developed shoots were further micropropagated by node cutting. For optimal micropropagation by node cutting, MS medium supplemented with 3.0mg/l Kn+0.5mg/l GA₃ was most effective. More than 75% of these micropropagated plantlets were successfully established and showed new leaf development under soil condition. This optimized meristem culture technique would be useful for developing uniform and virus-free clones of sweet potato in which viral diseases are predominant.

Keywords: *In vitro*, meristem culture, virus-free plant, micropropagation, sweet potato, *Ipomoea batatas*, viral disease

Abbreviations: BAP-6-Benzylaminopurine; Kn-kinetin; NAA- α -naphthalene acetic acid; PGR-plant growth regulator; TDZ-thidiazuron

Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam) belongs to the Convolvulaceae family, is among the world's most important, versatile, and underexploited food crops that ranks fourth among the food crops after rice, potato and wheat and seventh in the world in terms of total production (FAOSTAT, 2008). It is relatively drought tolerant, can grow on soils with limited fertility, provides good ground cover and often cultivated without fertilizer or pesticides (Ewel, 1990). It has remarkable pro-vitamin A qualities (Woolfe, 1992) and an important staple source of calories, and consumed by all age groups. Sweet potato ranks third among the 10 major crops of the world on a calorie per surface unit basis (Boukamp, 1985). These aspects are attractive to agriculturist, ecologists and economists interested in developing sustainable food production system in the tropics (Ewel and Mutuura, 1994; Hagemana et al., 1999). Sweet potato productivity is however, limited by a number of both biotic and abiotic

constrains. Viral disease is the most important limiting factor for sweet potato production. Worldwide, up to 20 different viruses have been identified to infect sweet potato (Loebenstein et al., 2003). Among them Sweet potato feathery mottle virus (SPFMV, genus *Potyvirus*, family *Potyviridae*) is found in all sweet potato growing areas while the others are localized to one or more geographic areas (Moyer and Salazar, 1989; Kreuze et al., 2000). The sweet potato virus disease (SPVD), usually caused by the dual infection of sweet potato feathery mottle virus (SPFMV) and sweet potato chlorotic stunt virus (SPCSV), is the most severe disease affecting the crop and can cause 56-98% yield loss (Gibson et al., 1998). SPVD epidemics have been, in many cases, associated with the disappearance of the once elite cultivars (Gibson et al., 1997).

Control of sweet potato feathery mottled virus (SPFMV) and sweet potato weevil is the most important strategy of International Potato Center (CIP)

in order to increase production and quality. But it is difficult to control these pests by using pesticides or other chemicals, thus calling for alternatives. Meristem-tip culture allows plants to be freed from other pathogens including viroides, mycoplasmas, bacteria and fungi (LaMotte and Lersten, 1972; Walkey, 1978; Kartha, 1984; Pierik, 1989; Grout 1999; Bhojwani and Razdan, 1996). Several reasons such as absence of plasmodesmata in the meristematic domes, faster cell division, competition between synthesis of nucleoproteins for cellular division and viral replication and presence of inhibitor substances make meristem tip useful source to obtain virus free plantlets. Another advantage is the genetic stability inherent in the technique. Plantlet production is from an already differentiated apical meristem and propagation from adventitious meristems can be avoided. Shoot development directly from the meristem avoids callus tissue formation and adventitious organogenesis, ensuring that genetic instability and somaclonal variation are minimized. Thus, uniform and clean seed derived from mericlones may be economically feasible for growers compared to conventional propagation. Benefit of meristem culture has been demonstrated for disease elimination in many crops including potato (Bittner et al., 1989), garlic (Conci and Nome, 1991), peanut (Morris et al., 1997), sugarcane (Balamuralikrishnan et al., 2002) and tomato (Alam et al., 2004). Reports on meristem culture in sweet potato are still limited (Frison and Ng 1981; Dagnino et al., 1991). Dong (1986) evaluated the effect of growth regulators callus induction and shoots formation from storage root explant. However this protocol suffers from high rate of microbial contamination during *in vitro* culture and is not suitable for virus-free stock production. Very high degree of genetic variation exist in the global sweet potato population that highly influence in their *in vitro* response (Rossel et al., 2001) Moreover, the published protocols are often not equally suitable even unsuitable for other cultivars. Our goal is to develop an economically viable diseases-free planting material production system through meristem culture in sweet potato. As the initial step, we evaluated a range of growth regulators to establish an optimized protocol for meristem culture and plantlet establishment.

Materials and methods

Primary establishment of shoot apical meristem in culture media

Sweet potato vines were collected from Tuber Crop Research Center, Agricultural Research Institute, Joydebpur and Regional Station, Bogra, was maintained in the Botanic Garden for collecting explants. Excised shoot tips collected from actively growing twigs were washed under running tap water and disinfected with 0.1% mercuric chloride solution containing approximately 0.02% Tween-20 [polyoxyethelen (20) sorbitan, oleate] for 6 min inside the running laminar air flow cabinet. Treated explants were washed 4-5 times with sterile distilled water to remove the effect of sterilizing agent. Shoot apical meristem

Table 1. Effect of different concentrations and combinations of Kn and GA₃ in MS medium for primary establishment of apical meristem of shoot tips from field grown plants. (Data were recorded after 4 weeks of inoculation)

PGR(mg/l)	Survival (%)	Average vigor
Kn		
1.0	47	0.56
2.0	56	0.67
2.5	64	0.62
3.0	50	0.58
GA₃		
1.0	36	0.51
1.5	44	0.58
2.0	50	0.60
3.0	47	0.56
Kn+GA₃		
2.0+0.1	58	0.60
2.0+0.5	75	0.79
2.5+0.1	61	0.67
2.5+0.5	66	0.71

consisting of the apical dome with one to two leaf primordia was isolated using sterile hypodermic needle and scalpel under a dissecting microscope (Olympus) as described previously (Alam et al., 2004). To avoid dehydration, isolated meristems (0.3-0.5 mm) were transferred quickly on the filter paper bridge in test tubes containing sterilized liquid MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of BAP, TDZ, GA₃ and Kn either singly or in combinations (Table.1). After 3-4 weeks, the developed meristems were subcultured on semi-solid medium with different levels of plant growth regulator for next 3-6 weeks for shoot elongation.

Mass multiplication and root formation

The meristem derived shoots (mericlones) were further multiplied by node cutting. The node segments were cultured at different growth regulators. In most cases the shoot multiplication was concomitant with rooting. Therefore, no additional supplementation was applied for root induction. In all cases, media were supplemented with 3% sucrose. The pH of the medium was adjusted to 5.7 followed by autoclaving at 121°C for 20 min (1.06 kg cm⁻²). For preparing semisolid medium, 0.8% agar (w/v), (BHD, England) was added after adjusting pH. Cultures were maintained at 25°C under a 16/8-h (light/dark) photoperiod with a light intensity of 48-50 μmol m⁻² s⁻¹ supplied by cool-white fluorescent lamps.

Acclimatization

Acclimatization of the mericlones was performed as described previously (Pal et al., 2007; Alam et al., 2009). Plantlets, with a well-developed root system, were washed carefully to remove agar and then transferred to pots containing sterile potting mix. Each pot was enclosed in a polyethylene bag after watering, and maintained in a growth chamber at 25±1°C under 16 h

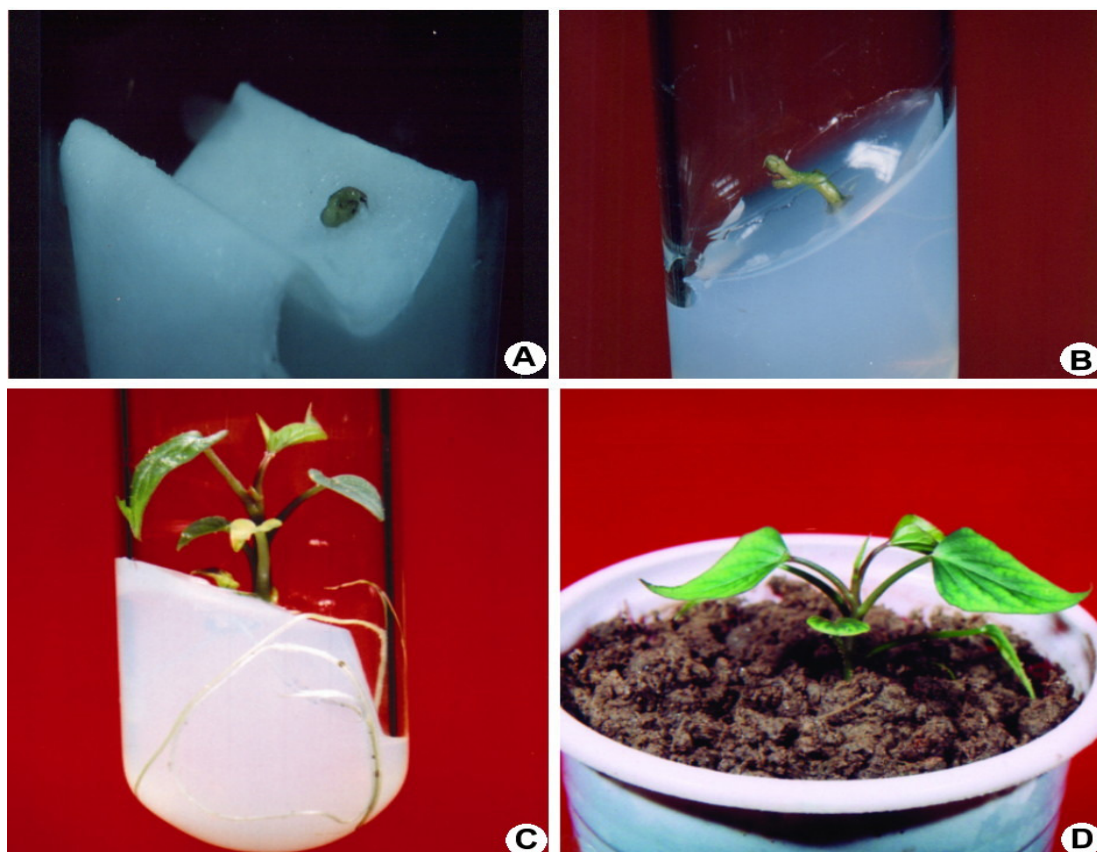


Fig 1. Representative picture of meristem culture and plantlet establishment in sweetpotato. (A) Development of isolated apical meristem (10-day old) on filter paper bridge in liquid MS medium. (B) Shoot with primary leaf development after subculturing in the semi solid medium. (C) Development of complete plantlet with root (D) Acclimatization of plantlets in the soil.

illumination ($145-150 \mu\text{mol m}^{-2} \text{s}^{-1}$) with fluorescent lamps. Bags were progressively opened weekly.

After 15-20 days of acclimatization had been completed, plantlets were transferred to field further growth.

Data recording

Percentage of meristem shows growth response, average degree of meristem vigor, number of shoots per explant and number of roots per shoot were recorded during meristem culture establishment and micropropagation stage. Average degree of meristem vigor was calculated from visual observation, using a hypothetical 0.00-1.00 scale; where, 0.00 = no growth, 0.25= poor growth, 0.5 = moderate growth, 0.75= good growth and 1.0 = excellent growth. Each *in vitro* treatment contains at least 12 replications and the entire *in vitro* experiment was repeated thrice.

Results and Discussion

Primary establishment of isolated meristems

Results on meristem culture in surface sterilized shoot tip in liquid medium are presented in Table.1. Initial growth of the cultured meristem started within 6-10 days by increasing in size (vigor) and in color (greenish) (Fig 1A). Growth was continued to shoot

(and sometimes root) development, resulting primary establishment of meristem. On this trait, MS medium supplemented with 2.0 mg/l Kn plus 0.5mg/l GA₃ showed the most vigorous response. In this combination about 75% of excised meristems responded with an average vigor of 0.79. Meristems were failed to develop further when cultured in growth regulators-free medium. A varied degree of unexpected callus formation instead of shoot formation was observed when different concentrations of BAP or TDZ (ranged from 0.05 to 1.5 mg/l) were used in the medium and considered unsuitable for this cultivar (data not shown). Regarding meristem size, smaller than 0.3mm did not survive and 0.3 to 0.5 mm sized meristems were used. Isolated meristem cultured in the liquid medium was beneficial since most meristem failed to respond in semisolid medium. Being a tiny and free of conducting tissues, liquid culture medium is beneficial for growth and development of isolated meristem as found in our experiment and others (Elliott, 1969; Alam et al., 2004). The advantage of liquid medium lies in easier availability of water and dissolved nutrients to the entire surface of the explants. Plant regeneration from meristem *in vitro* usually requires exogenous hormonal supplementation of culture medium. A combined use of Kn and GA₃ gave most promising results in our study. However, most other reports on sweet potato meristem culture used NAA, IAA, BAP and 2,4-D for establishment of primary meristem (Hettiarachchi,

Table 2. Effect of different combinations of Kn and GA₃ in MS medium for multiplication of virus-tested mericlones using nodal explants. Data were recorded after 4 weeks of inoculation.

PGR(mg/l)	No. of Shoot ($\bar{x} \pm SE$)	Shoot length ($\bar{x} \pm SE$)	No. of Root ($\bar{x} \pm SE$)
Kn			
2.5	3.12±0.12	4.76±0.12	13.12±0.60
3.0	3.81±0.19	4.85±0.17	16.31±0.31
4.0	2.88±0.22	5.17±0.15	15.48±0.75
Kn+GA₃			
2.5+0.5	4.10±0.21	5.30±0.13	13.72±0.50
3.0+0.5	4.94±0.20	5.91±0.14	13.88±0.71
3.0+1.0	2.76±0.11	4.22±0.19	11.09±0.61
Kn+NAA			
2.0+0.5	2.19±0.17	3.65±0.12	16.57±0.45
3.0+0.5	2.07±0.11	4.31±0.16	18.10±0.28
3.0+1.0	1.60±0.14	3.47±0.13	18.91±0.17

1988; Garrido and Casares, 1989; Dagnino et al., 1991). The differential response to the treatments over the previous works is probably due to genotypic variation exist in sweet potato germplasm since *in vitro* response is affected by genotype in many species (Mamidala and Nana, 2009). This is probably due to genotypic variation. Considerable number of isolated meristem died even in the suitable media, probably due to dissection injury. About 25-40% death rate was described as a normal phenomenon in most of the published protocols.

Shoot and root development from the primarily established meristem on semisolid medium

Around 75-80% of the meristem derived tiny shoots shows further development when transferred to semisolid medium containing 2.5 mg/l Kn plus 0.5 mg/l GA₃, GA₃ (2.0 mg/l) or Kn (2.5 mg/l) (data not shown). Considering growth response, use of 2.5 mg/l Kn+0.5mg/l GA₃ is recommended for proper shoot growth from primary shoot (Fig 1B). Combined use of Kn and GA₃ in MS medium was reported earlier for plantlets development from meristem-tip culture in sweet potato (Love et al., 1989). Use of BAP and GA₃ was also reported (Dong, 1986; De-Andrade and De-Andrade, 1995). Failure of growth in MS₀ justified the necessity of growth regulators in this stage of development. For a virus-free seed program, at this stage, part of the developed plantlets can be used for virus assay before going for massive micropropagation program.

Micropropagation of mericlones

Single nodal cultures of meristem-derived plantlets (Fig 1C) responded differently to the composition of the micropropagation medium. All cultures developed shoots in the culture media tested. Among those, MS medium supplemented with 3.0 mg/l Kn plus 0.5 mg/l GA₃ were found very effective in promoting shoot number and length. On the other hand, maximum

number of roots was obtained in the medium containing 3.0 mg/l Kn plus 1.0mg/l NAA. More or less similar observations are also reported by González et al., (1999). Combinations of Kn with NAA for adventitious shoot development were reported by many workers (Lu et al., 1995; González et al., 1999). These combinations were associated with higher number of roots as observed by Dagnino et al. (1991). For high frequency root formation, combination of 3.0 mg/l Kn + 0.5 mg/l NAA was most effective. Although, the combination 3.0 mg/l Kn+0.5 mg/l GA₃ also produced relatively less number of roots but it was found sufficient for acclimatization and subsequent establishment in soil. About 26 plants were established among the 33 transplanted to soil e (Fig 1D). So for high frequency micropropagation of meristem derived plantlets, MS medium supplemented with 3.0 mg/l Kn+0.5 mg/l GA₃ is recommended.

In conclusion, plant regeneration from shoot apical meristems was best enhanced using liquid MS medium added with 2.0mg/l Kn and 0.5mg/l GA₃. Shoot and root development proceeded without an intermediate callus phase. The meristem-derived plantlets were effectively and rapidly micropropagated using single nodal segments within 6-8 weeks of meristem inoculation. The potential of meristem derived plantlets of sweet potato to be used in soil condition was investigated by transferring the plantlets to soil pots in a growth chamber. After 3 weeks of hardening under culture-room condition, more than 75% of the regenerants survived and showed new leaf development. Meri- clones are generally considered as virus-free plants. Upon proper virus assay, these clones would be used as virus-free clonal stock. Research on the feasibility of this protocol for disease-free stock production is currently in progress.

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