# PLANT REGENERATION FROM LEAF AND STEM EXPLANTS FROM TWO SWEET POTATO (Ipomoea batatas L. Lam.) CULTIVARS

✉ Rolando García González, Danalay Somontes Sánchez, Jesús Mena Campos, Eulogio Pimentel Vázquez, Zurima Zaldúa Guerra, Alina López Quesada, Rolando Morán Valdivia, Melba García González

División de Plantas. Centro de Ingeniería Genética y Biotecnología. Camagüey. AP 387, CP 70100. Camagüey, Cuba. Tel: (53-322) 61295; Fax: (53-322) 61587; E-mail: invest@cigbcam.cigb.edu.cu

**ABSTRACT**

An efficient protocol for organogenic regeneration of two sweet potato *(Ipomoea batatas* L.) elite cultivars, was developed. Several growth regulator combinations and explant sources were tested, so an efficient protocol for plant regeneration was established. Out of 151 plant growth regulator combinations, three were able to induce the highest percentage of shoot and root formation for both cultivars. The first medium was supplemented with 1.0 mg/L naphthalene acetic acid (NAA) and 0.1 mg/L benzylaminopurine; the second, with 1.0 mg/L paclobutrazol and 0.1 mg/L NAA; and the third, with 0.5 mg/L indol-3-acetic acid.

*Keywords:* growth regulators, *Ipomoea batatas*, plant regeneration, sweet potato, tissue culture

*Biotecnología Aplicada 1999;16:11-14*

**RESUMEN**

En este trabajo reportamos la obtención de una metodología para la regeneración de dos variedades comerciales de boniato *(Ipomoea batatas* L.). Para realizar nuestro estudio, ensayamos diferentes combinaciones de reguladores de crecimiento, así como distintas fuentes y tipos de explantes. Sólo tres combinaciones de las 151 variantes ensayadas fueron capaces de inducir la formación de brotes aéreos y raíces con una alta frecuencia. El primer medio corresponde a la combinación de ácido naftalenacético (ANA) a una concentración de 1,0 mg/L y benzilaminopurina a 0,1 mg/L en medio MS; la segunda combinación correspondió a la adición de 1,0 mg/L de paclobutrazol y 0,1 mg/L de ANA al medio MS; y la tercera variante, con resultados satisfactorios, fue el medio MS suplementado con 0,5 mg/L de ácido 3-indolacético.

*Palabras claves:* boniato, cultivo de tejidos, *Ipomoea batatas*, regeneración de plantas, reguladores del crecimiento

## Introduction

Sweet potato *(Ipomoea batatas* L.) ranks seventh among all food crops worldwide, with an annual production of 115 million metric tons. Of the root and tuber crops, the sweet potato ranks third in acreage (7.9 million ha) behind the potato and cassava [1]. Sweet potato is grown in more than 100 countries and among the world's root and tuber crops, it ranks second in importance [2]. It is consumed as a fresh vegetable (roots, petioles, leaves and stems), staple food, snack food and it is also used for industrial starch extraction and fermentation [3-6]. Sweet potato is industrially dehydrated [7] and used as an important component of bread flour [8].

Although the sweet potato is one of the most important plants in the world, biotechnological work on it has lagged behind. In recent years a few groups have reported their experience in the molecular biology manipulation of sweet potato to increase nutritional quality [9] and pest resistance [10]. Others have been working on its cryopreservation [11], protoplast isolation and regeneration [12, 13], and its regeneration and transformation from roots, petioles, stems and leaves [14-17].

Sweet potato is more tolerant and needs less nutrients from the soil than most of the crops, being therefore, one of the most cultivated crops in developing countries. However, the increase in production is limited due to the severe damages caused by pests and diseases. In many regions where sweet potatoes are produced in low-input agricultural systems, insect related losses may often reach 60-100% [18].

The sweet potato weevil *(Cylas* spp.) is the major biological antagonist of sweet potatoes worldwide. Controlling is quite difficult and the improvement programs developed have only been partially successful. The improved cultivars do not show a stable performance in different ecological conditions; most of them depend on the soil structure or the physiological and botanical features of the cultivar rather than on the genetic production of chemical defenses against the pest [19, 20]. Both of the above mentioned reasons and the lack of any strong conventional genetic improvement program to obtain pest resistance or to increase its nutritional quality, make the sweet potato an important target to be modified by biotechnological tools.

It is recalcitrant to regeneration and transformation because each cultivars shows different responses to *in vitro* treatments. Several protocols reporting sweet potato regeneration [10, 14-16, 21] were tested, in order to establish an efficient transformation methodology, but they failed to work for our cultivars under our conditions. Every cultivar vary widely in their response to tissue culture and plant regeneration [14, 15]. Hence, the genotype is one of the most important factors affecting the evolution of the *in*

✉ Corresponding author

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*vitro* culture, regeneration and transformation responses [14, 15, 21, 22].

The latest and most successful protocol obtained for the regeneration and *Agrobacterium tumefaciens* mediated transformation of sweet potato was reported by Newell, *et al.* from the University of Bath in 1995 [10].

The target explants used for the Newell group were tuberous roots grown in greenhouses where many conditions were tested to induce regeneration from the meristematic bud-like centers (MBLC), potentially existing in the tuberous root tissue. Transgenic shoots were obtained following this methodology at 16 weeks of culture after the transformation step. The shoots showed to be transformed when tested. Newell's paper was the first report of the transformation of commercial sweet potato cultivars with an economically interesting gene; the previous papers were only on the introdutcion of genes into the sweet potato genome. However, Newell's methodology is too difficult to reproduce because the target explant used is very hard to clean and to adapt to the *in vitro* culture conditions. Additionally disadvantageous is that the protocol reported by Newell took a long time to regenerate transgenic shoots. The regeneration and transformation frequencies shown in the article were about 5%, but the number of explants manipulated during the experiments were very high.

The aim of this paper is to establish an efficient methodology for sweet potato regeneration to make the following transformation steps possible. The target explants used were leaves and stems. The first transgenic shoots via organogenesis were obtained three to four weeks after cultivation on the regeneration medium. Two very important cultivars were studied in our experiments: Jewel and CEMSA-78354. Our study was separated into two steps. As a first step different growth regulator combinations and three sources of explants were tested in order to establish an efficient medium for shoot induction. The second step included certain factors that could affect the transformation such as: induction of *vir* genes, time of cocultivation, temperature, etc, which are currently tested.

## Materials and Methods

### Vegetable material

Two genotypes were used during all the experiments: Jewel, a very important commercial genotype cultivated in North and South America, and CEMSA-78354, the most commonly used in Cuba. Both were supplied by the collection of the Instituto Nacional de Investigaciones en Viandas Tropicales (INIVIT) in Santo Domingo, Cuba. The *in vitro* plants are kept in an MS [23] solid medium supplemented with Sucrose 3%, IAA 0.5 mg/L, agar-agar 6.0 g/L and pH 5.6-5.7 adjusted before autoclaving.

### Regeneration conditions

Leaves and stems were taken from young plants grown in glass culture tubes containing 7 mL of the MS medium supplemented with sucrose 3%, nicotinic acid 0.5 mg/L, thyamine-HCl 0.1 mg/L, piridoxine-HCl 0.5 mg/L, glycine 2 mg/L, myo-Inositol 100 mg/L, indol-3-acetic acid 0.5 mg/L (aidded prior autoclaving). The pH was adjusted to 5.6-5.7 before autoclaving (20 min; 121 °C; 138 kPa). The *in vitro* plants were kept for six weeks at 25 °C in a 12/12 photoperiod, 66% relative humidity.

All experiments were carried out in 10 cm petri dishes containing 25 mL of each regeneration medium. All the experiments were randomly set up in six replicates for each growth regulator combination. Each replicates consisted of 6 leaves and 10 stems. The explants were incubated at 25 °C and in a 12 h photoperiod and all were subcultured in a fresh medium every 15 days. The final evaluation of the experiment was performed after six weeks. All treatments were assessed according to their influence on the tissues' responses to regeneration and differentiation, following the criteria described below.

The basal medium for all studies was the MS basal salt mixtures and vitamins supplemented with 3% sucrose and solidified with agar noble Difco (Difco Laboratories Inc.). The pH was adjusted to 5.6-5.7 before autoclaving (20 min; 121 °C; 138 kPa). All growth regulators were added prior to autoclaving, except zeatin riboside, which was added filter-sterile to the cooling medium.

We used the whole leaf trying to keep a 2-5 mm piece of petiole; the leaves were placed and expanded on the media. Stems were removed very carefully, trying not to extract any vegetative bud; there was no regular size for the stem pieces, since we used the internodal section without considering its size.

### Evaluation criteria

To select the best regeneration conditions we studied the influence of each treatment on: calli formation, root regeneration and shoot emission. We calculated the regeneration efficiency of direct shoots as follows:

$$RE = (RS / TNE) \times 100$$

where:

*RE:* percentage of regeneration efficiency of direct shoots

*RS:* total number of regenerated shoots

*TNE:* total number of tested explants

The term regeneration frequency was introduced to determine the functional shoot or root emission per explant. It was calculated as follows:

$$RF = NRP / TNE$$

where:

*RF:* regeneration frequency per explant

*NRP:* number of rooted functional shoots

*TNE:* total number of tested explants

### Regeneration studies on solid medium

Sweet potatoes are reported to have a genotype dependent response both to organogenesis and somatic embryogenesis. Therefore a wide number of growth regulators have been tested for their influence on several sweet potato genotypes and they have shown different patterns in calli formation and shoot and root emission [10, 14-16]. Several growth regulator combinations reported as having different effects on sweet

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potato regeneration were tested. Combining auxins and cytokinins or using them alone in the medium were the basic treatments tested to achieve high root and shoot emission.

### Growth regulator combinations

a) *First experiment.* Thirty five combinations of two growth regulators at different concentrations were tested: [indol-3-acetic acid (IAA) (0.0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L, 1.0 mg/L, 1.25 mg/L, 2.0 mg/L) and zeatin riboside (0.11 mg/L, 0.22 mg/L, 0.5 mg/L, 0.75 mg/L, 1.0 mg/L)]. The explants were cultured on these combinations for six weeks at 25 °C and a 12/12 h photoperiod. All the explants were subcultured in a fresh medium every 15 days. Partial evaluations every 10 days and a final evaluation at the end of the experiments, were performed. The general culture conditions were the same for the next experiments.

b) *Second experiment.* Another cytokinin growth regulator was tested combined with IAA, instead of zeatin. The IAA levels and the conditions for this study were the same as for the first experiment. Kinetin riboside was used as cytokinin at different concentrations (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L). 28 combinations were used.

c) *Third experiment.* 6-benzylaminopurine (BAP) at 0.25 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L and 3.0 mg/L was used combined with AIA at similar levels to the preceding experiments. The experimental conditions were stable for this and the next two experiments. 35 combinations were tested.

d) *Fourth experiment.* Twenty variants were tested in this experiment. Auxin was changed for this study and BAP was kept at the same levels as the above cytokinin. Naphthalene acetic acid (NAA) was added at: 0.1 mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L.

e) *Fifth experiment.* NAA was combined with kinetin at 0.1 mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L. Six variants were studied from the combinations of these regulators at the concentrations described.

f) *Sixth experiment.* A new growth regulator, unreported for sweet potato tissue culture, was used in this study. Paclobutrazol (PPP) is a chemical that blocks the giberelic acid pathway, thus, dominance exclusion is expected when it is supplied to the plant. For sweet potato this is a very common phenomenon. In previous studies we demonstrated that the first shoots and roots regenerated from leaf explants restrained other shoot and root formations on the same explants. When the first regenerated shoots were removed from the explants, other shoots or roots were formed from the original explants. In this experiment NAA at 0.0 mg/L, 0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L and PPP at 0.0 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, were combined. PPP was added to the medium before pH was adjusted to 5.6-5.7; then it was coautoclaved at 120 °C and 138 kPa for 20 min. L-cysteine was added to 40 mg/L and ascorbic acid to 80 mg/L was added after sterilization, to avoid plant tissue oxidation. The explants were kept on these combinations for six weeks and were then evaluated. PPP and NAA combinations resulted in 20 variants.

## Results and Discussion

It was difficult to analyze all the data collected after such a large combination of variants. The number of shoots and roots regenerated, and the regeneration frequency (shoots or root regenerated on the total number of explants), were considered for the selection of a competent condition for sweet potato regeneration. As in all the regeneration and transformation protocols it is very important to have high plant conversion in order to increase the likelihood of transformed plants.

Leaves had a higher regeneration frequency compared to stem. Therefore, we consider that leaves are suitable target explants in the transformation studies based on this organogenesis mediated protocol. The most potential area for shoot and root formation was just above the cut piece of petiole on the leaf where a small callus was formed before the emission of roots or shoots, or both.

The stem explants were able to form non-embryogenic calli on the cut side of the explants but early organogenic shoots appear before these calli and they were always regenerated from the meristematic area placed in the nodal section. The CEMSA-78354 stems were harder to manipulate than the Jewel since in the former, the internodal segments are very short and the vegetative buds may have not been completely removed. As a result of this handicapped shoot emission, more than 95% were obtained for the CEMSA-78354 and 70% for the Jewel emissions, on several media from both cultivars (MS + IAA 0.5 mg/L + sucrose 3%; MS + sucrose 3% + BAP 0.1 mg/L + NAA 1.0 mg/L; MS + sucrose 3% + Kin 0.1 mg/L + IAA 0.5 mg/L). However, it is believed that this was due to some remaining meristematic and differentiated tissues from the buds that continued developing into normal plants under the growth regulator effects. Although not yet demonstrated, the classical organogenesis and bud development was observed only a week after culturing the explants under the above mentioned conditions.

Remarkably, the influence of auxin to induce direct organogenesis was observed to be greater than that one of the cytokinins. More stimulation for non embryogenic calli formation were usually found when the explants were treated with cytokinins (even at low concentrations) compared to auxins. In some cases shoots were achieved by giving moderated auxin treatments (IAA 0.5 mg/L; NAA 0.1 mg/L). There is no previous reference of using auxin based stimulation alone to induce plant regeneration in sweet potato, especially for these cultivars.

The CEMSA-78354 cultivar was more difficult to regenerate than the Jewel. This cultivar was induced to form calli under low levels of any kind of growth regulator. The calli were compact and hard to manipulate with blades, they were green throughout the area that was in full contact with the medium, but the cells on the top were always white. Root emission and sporadic shoot formation were obtained as well, but at a very low frequency on a growth regulator free MS

medium. This must be due to the endogenous growth regulators for this cultivar. Secondary roots were formed profusely in 127 treatments assayed and it is thought to be a very important fact blocking shoot formation. When the first roots or shoots emitted were separated from the original explants, new roots or shoots were formed again.

Shoot induction both from the original explant and the regenerated root were achieved on several combinations of growth regulators during the regeneration study. However, none was above 20% of regeneration, except for three of the growth regulator combinations studied on which the following observations were based. The most significant results were obtained for the following three combinations (Tables 1-3).

The direct shoots are formed both by organogenesis or embryogenesis, from the original explants. Indirect shoots are regenerated from *de novo* roots formed from the original explants. More than one shoot were usually obtained from the regenerated roots for the Jewel cultivar. Of greater importance was the indirect shoot formation in CEMSA-78354: 92.8% of the shoots obtained were induced from the regenerated roots.

Certain authors have reported the formation of MBLC [10] in the tuberous roots, but none of the studies reported mentioned this phenomenon for the regenerated roots. These indirect shoots started as organized dark red cells on the surface of the roots and were rapidly differentiated into plants by organogenesis. The shoots formed were capable of rooting in the MPM propagation medium.

The influence of PPP on sweet potato regeneration was assessed. As the elimination of dominance is expected when PPP is in the medium, the frequency of shoots per explant can be increased. This has been proven for both cultivars. The number of roots emitted per explant was over 5 when the explants were treated with a concentration above 0.5 mg/L of PPP.

On the other hand, the number of roots forming indirect shoots was higher for these treatments. However, severe tissue oxidation and non-functional shoots were usually observed when the PPP concentration was over 1 mg/L, specially for CEMSA-8354.

As already stated, the CEMSA-78354 cultivar was more difficult to regenerate than Jewel. All the explants tested were easily induced to form calli under low levels of any kind of the growth regulators tested. Root emission was achieved even on growth regulator-free MS medium. Secondary roots were formed profusely and they are thought to block shoot formation in many of the treatments assayed; in fact, the emission of new roots and shoots was stimulated when the first roots and shoots were cut out from the explant. Besides, a high number of explants developed more than one shoot, but only one was able to grow unless they were detached from each other. When the most developed shoots were taken from the original explant, the shortest ones started to develop into normal plants. Although the dominance effect was maintained, it was less important than that induced for the first shoot.

Again, the differences between two sweet potato cultivars was shown, now in all of our experiments, which supports the previous studies that were limited to several conditions for one cultivar [24] and are limited for other cultivars.

Finally, it is underlined that no embryogenic response was observed in any of the studies. All the shoots and roots were achieved by direct or indirect organogenesis. Embryogenesis for sweet potato has always been obtained giving long 2,4-D treatments [10, 12, 23], but it has not worked for our cultivars. Calli started growing very quickly and they were formed by globular structures during the second week for the earliest treatment and at the sixth for the last. However, these structures became undifferentiated two weeks after their formation and they regenerated into a root or a shoot in all the experiments without completing the last embryogenic steps.

The regenerated shoots were always planted in the MPM medium for rooting and they developed whole plants for both cultivars. These plants did not differ in their phenotype with the wild *in vitro* cultured plants adapted to greenhouse conditions.

Table 1. Regeneration on a BN-3 medium [MS + sucrose (3%) + BAP (0.1 mg/L) + NAA (1.0 mg/L)]. Note that the Jewel cultivar behaved better than the CEMSA-78354.

| Cultivar | Direct shoots (%) | Rooting | Indirect shoots | Regeneration frequency |
|---|---|---|---|---|
| Jewel | 68.90 | + + + | 100.00 | 0.70 |
| CEMSA-78354 | 13.75 | + + + | 3.00 | 0.14 |

+: moderated; + +: good, + + +: high

Table 2. Regeneration on a PN-2 medium [MS + sucrose (3%) + PPP (1.0 mg/L) + NAA (1.0 mg/L)].

| Cultivar | Direct shoots (%) | Rooting | Indirect shoots | Regeneration frequency |
|---|---|---|---|---|
| Jewel | 85.00 | + + + | 100.00 | 0.85 |
| CEMSA-78354 | 27.50 | + + + | 12.00 | 0.27 |

+: moderated; + +: good; + + +: high

Table 3. Regeneration on a MPM medium [MS + sucrose (3%) + IAA (0.5 mg/L). Note that the frequency of shoot per explants for Jewel was above 2.

| Cultivar | Direct shoots (%) | Rooting | Indirect shoots | Regeneration frequency |
|---|---|---|---|---|
| Jewel | 200.00 | + + + | 100.00 | 3.0 |
| CEMSA-78354 | 22.50 | + + + | 12.50 | 0.3 |

+: moderated; + +: good; + + +: high

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*Received in August, 1997. Acepted for publication in June, 1998.*