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Embryo-like structures and root regeneration induced by 2, 4-dichlorophenoxyacetic acid in twenty African sweet potato cultivars

Abel Sefasi[1]*, Marc Ghislain[3], Andrew Kiggundu[4], Gorrettie Ssemakula[2], Runyararo Rukarwa[1], Robert Mwanga[5], Jan Kreuze[3] and Settumba Mukasa[1]

[1]School of Agricultural Sciences, Makerere University, Box 7062, Kampala, Uganda.
[2]National Crops Resources Research Institute, Box 7084, Kampala, Uganda.
[3]International Potato Center, Box 1558, Lima 12, Peru.
[4]National Agricultural Research Laboratories Institute, Kawanda, P. O. Box 7065, Kampala, Uganda.
[5]International Potato Center, Box 22274, Kampala, Uganda.

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Embryo-like structures were induced from 'whole leaf' explants of sixteen of the twenty investigated sweet potato cultivars on Murashige and Skoog (1962) medium supplemented with 2,4 Dichlorophenoxyacetic acid (0.2 mg/L) at the Makerere University Agricultural Research Institute Tissue culture laboratory. Shoot and root regeneration was possible in the non-African cultivar, Jonathan, whereas only root regeneration was successfully induced in five African cultivars. Cultivar type had a highly significant ($P < 0.001$) effect on frequency of embryo-like structures and efficiency of both shoot and root regeneration. The embryo-like structures induced could be useful for initiation of cell suspensions to enable genetic transformation of African cultivars. The method for *de novo* regeneration of roots has potential application in the regeneration of plants or hairy root cultures for cultivars that are recalcitrant to shoot regeneration.

Key words: 2, 4-dichlorophenoxyacetic acid, cultivar, *in vitro* recalcitrance, regeneration efficiency, somatic embryogenesis.

INTRODUCTION

Sweet potato (*Ipomoea batatas* (L.) Lam.) is mainly cultivated for its expanded edible roots which contain high carbohydrate and beta-carotene, depending on cultivar. The total sweet potato production for the world is 103.1 million metric tons (USDA, 2015). However, the potential production for sub-Saharan Africa is not

*Corresponding author. E-mail: asefasi@bunda.luanar.mw; abelsefasi@yahoo.co.uk. Tel: +265-0998552090.

attained, as yields are very low at about 5 t/ha (Luo et al., 2006). The low yields are mainly due to various challenges including high incidence of virus disease and increasing attacks by sweet potato weevils (Fuglie, 2007).

For a long time, traditional breeding has contributed to the improvement of crop traits. Nevertheless, due to the high male sterility, self and interspecific incompatibility and hexaploid (2n = 6x = 96 chromosomes) nature of sweet potato, conventional hybridization is still difficult, and hybrid progenies undergo segregation leading to loss of many valuable traits (Yang et al., 2011). Genetic transformation is a promising tool to improve sweet potato traits like nutrition and resistance to weevils and viruses (Ghislain et al., 2008; Kreuze et al., 2008; Shimada et al., 2006). Genetic transformation enables access to genes from unrelated species thereby enabling breeders to improve plants with traits that are not available in the crop germplasm (Ghislain et al., 2008).

Efficient development of transgenic plants requires optimal transformation and regeneration conditions. Although, successful induction of somatic embryogenesis has been reported in sweet potato, most of the published protocols are cultivar-specific (Santa-Maria et al., 2009). Low embryogenic frequencies and complete recalcitrance has been reported for most important and adapted sweet potato cultivars (Al-Mazrooei et al., 1997; Liu et al., 2001; Zang et al., 2009), particularly for African cultivars (Luo et al., 2006). The non-African cultivars that have been transformed so far, including Jewel (Luo et al., 2006), Huachano (Kreuze et al., 2008), White Star (Gama et al., 1996), Kokei 14 (Otani et al., 1998), Yulmi (Choi et al., 2007) and Lizixiang (Yu et al., 2007), can only be used for research purposes in Africa. These are not farmer-preferred cultivars due to several drawbacks, such as low dry matter and vulnerability to diseases and pests (Yang et al., 2011). Hence, the development of an efficient *in vitro* regeneration and transformation method for farmer-preferred sweet potato varieties in sub-Saharan Africa is important and urgently required.

The objective of the study reported here was to screen a large number of farmer-preferred sweet potato cultivars for the response to somatic embryogenesis induced by 2,4-Dichlorophenoxyacetic acid (2,4-D). Published reports show that there is a correlation between the quality and quantity of embryo-like structures and subsequent plant regeneration in sweet potato (Al-Mazrooei et al*.,* 1997).

MATERIALS AND METHODS

Twenty farmer-preferred Ugandan sweet potato cultivars and one USA cultivar, Jonathan, were used in this study. These cultivars as shown in Table 1 were selected based on preferred attributes among consumers in Uganda, including high dry matter content (Mwanga et al., 2007; Mwanga et al., 2001). The non-African cultivar, Jonathan, was previously reported to regenerate through somatic embryogenesis (Cipriani et al., 1999). The cultivars were grown in a screenhouse at Makerere University Agricultural Research Institute, Kabanyolo. Vines from the screen house grown plants were grafted on *Ipomoea setosa,* an indicator plant for virus infection (Figure 1). Cuttings from those sweet potato plants corresponding to scions whose indicator plants were asymptomatic were used in establishment of virus-free *in vitro* cultures.

Cuttings containing five to eight nodes were taken from the greenhouse to the laboratory. The cuttings were washed for fifteen minutes with flowing tap water and then immersed for 20 min in 39% (v/v) commercial JIK containing 3.85 % sodium hypochlorite (NaOCl) and 0.03% Tween 20®. Thereafter the vines were submerged in 70% ethanol for 2 min. After surface sterilization, the vines were transferred to sterile water and rinsed three times. They were then each cut into one or two nodes and inserted into sweet potato propagation medium.

The propagation medium was composed of MS (Murashige and Skoog, 1962) salts premix (4.3 g l^{-1}), sucrose (30 g/L), myo-inositol (0.1 g/L), 5 ml/L sweet potato vitamin stock comprised of 40 g/L ascorbic acid, 20 g/L L-arginine, 4 g/L putrescine HCl, 0.01 mg/L gibberellic acid (GA_3) and 0.4 g/L calcium pantothenate (Kreuze et al., 2008). All reagents used in this study were sourced from Sigma-Aldrich. The medium was adjusted to pH 5.8 before adding 3 g/L phytagel and autoclaved at 121°C for 15 min under 15 kPa.

Four-week-old whole leaves (petiole with lamina) from the *in vitro*-grown plants were placed on callus induction medium (CIM) under dark conditions at 28°C. The cut end of the petiole was slightly pushed into medium. CIM was made of 4.3 g/L MS premix (without vitamins), 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 ml/L Vitamins stock (0.5 mg/ml nicotinic acid, 0.1 mg/ml thiamine and 0.5 mg/ml pyridoxine), 100 mg/L myo-inositol, 30 g/L sucrose and 7 g/L agar. The cultures were transferred to fresh media every two weeks. A total of ten to fifteen explants were used per cultivar in each petri dish, and this was replicated three times and arranged in a completely randomized design. After twelve weeks the callus formed was transferred to embryo initiation medium (EIM) under a photoperiod of 16 h, regardless of it being embryogenic or non-embryogenic. EIM was made as CIM but 2, 4-D was replaced with 1 mg/L abscisic acid (ABA). After four weeks on EIM, the callus was transferred to plant regeneration medium devoid of plant growth regulators. Fully developed plants were transferred to soil in a screen house where they developed normally.

Data on number of explants forming callus and those forming embryo-like structures were collected after twelve weeks. Data on shoot and root regeneration was collected every week since some callus formed roots within three weeks of culture initiation. Cultures were discarded after twenty-four weeks since at this stage most calli lost their embryogenic capacity and could not regenerate shoots. Frequency data was transformed using the arcsine square root before analysis to stabilize the variance. The table presented in this paper shows non-transformed data expressed as percentages. GenStat statistical package was used to analyse the effect of cultivar on total callus proliferation and formation of embryo-like structures, which was performed using general linear model of analysis of variance (ANOVA) at 5% level of significance. Mean values were compared using the least significance difference (LSD) method at the 5% level.

RESULTS

In the present study, explants placed on callus induction medium responded by producing callus within three days. The callus was not only on the wounded tip of the petiole but also on the intact leaf lamina (Figure 2B). Two types of callus were induced; embryogenic and non-embryogenic. The embryo-like structures were bright

Table 1. Some attributes of Ugandan cultivars selected for *in vitro* regeneration and genetic transformation.

Cultivar	Flesh color	SPVD resistance	Maturity (Days)	Dry matter (%)	Year of release and specific location of collection/release in Uganda
Kakamega	Intermediate orange	M	135	33	2004 in Uganda. Kenya landrace
NASPOT 1	Cream	M	135	32	1999 (bred clone)
New Kawogo	Cream	R	140	32	1995 (Landrace), Germplasm (1988)
Bwanjule	White/Cream	M	135	30	Landrace/ 1995, Germplasm (1988)
Araka	Cream/white	F	105	32	Landrace: Germplasm 2005
NASPOT 8	Pale orange	M	120	32	2007
Dimbuka-Bukulula	Cream	S	128	32	2001, Germplasm from Masaka
Silk omupya	White	R	100	34	Germplasm from Pallisa
Kyebandula	Cream	M	120	32	Landrace: Germplasm -Mbale
Munyeera	Cream	R	165	33	Landrace: Germplasm from Mpigi
Ssemanda	Cream/white	R	120	33	Landrace: Germplasm from Mpigi
Kisakyamaria	Cream	S	120	32	Landrace: Germplasm from Mbarara
Luwero Silk	Cream	S	120	34	Landrace: Germplasm -Luwero
Namusonga	Cream	F	120	34	Landrace: Germplasm from Pallisa
Magabali	Cream	R	165	33	Landrace: Germplasm-Kabale
Kigaire	Cream	M	120	32	Landrace: Landrace Soroti/Serere
Nyidoyamulalo	Cream	R	120	32	Landrace: Germplasm-Kamuli
UNK-Luwero 2 (Uknown)	Cream/white	S	120	33	Landrace: Germplasm -Luwero
UNK Luwero (Unknown)	White	M	165	30	Landrace: Germplasm -Luwero
Jamada	Cream	M	150	32	Landrace: Germplasm -Luwero

Note: all cultivars are adapted to Uganda with potential for adoption in other areas of East Africa. SPVD sweet potato virus disease resistance M = Moderate resistance, R = resistant, S= Susceptible, F=Fair, Dry matter content (%) = per cent of fresh weight.

Figure 1. Preparation of plant material for somatic embryogenesis experiments. **A**, sweet potato cultivars growing in greenhouse ready for bio-indexing. **B,** Bio-indexing of sweet potato (scion) by grafting on *Ipomoea setosa* (rootstock) indicator plants. **C,** *In vitro* cultures growing from virus-free and decontaminated plants. **D,** "Whole leaves' used for callus induction on medium supplemented with 2, 4-D.

yellow or orange in colour and compact in appearance, while non-embryogenic callus was white and friable and proliferated earlier and more rapidly than embryo-like structures. The embryo-like structures were induced in sixteen of the twenty investigated African cultivars cultured on media with 0.2 mg/L 2,4-D. Cultivar type had a highly significant ($P < 0.001$) effect on embryo-like structures induced (Table 2). The frequency of embryo-like structures ranged from zero to 25% depending on cultivar (Table 2). Embryo-like structures started developing within six weeks of initiation of cultures.

Somatic embryos of cv. Jonathan developed into shoots after three weeks on ABA-containing media. Cultivar type also had a highly significant ($P < 0.001$) effect on the ability to regenerate shoots (Table 2). Regeneration of roots was achieved from callus of five African cultivars. Contrary to the regeneration of shoots which occurred through somatic embryogenesis, the regeneration of roots appeared to be so through organogenesis as they were formed directly from callus without somatic embryos. In addition, the roots were regenerated earlier than shoots, sometimes, as early as three weeks after culture initiation on 2, 4-D-containing media. The emergence of new roots was sustained throughout the culture period. The type of cultivar showed

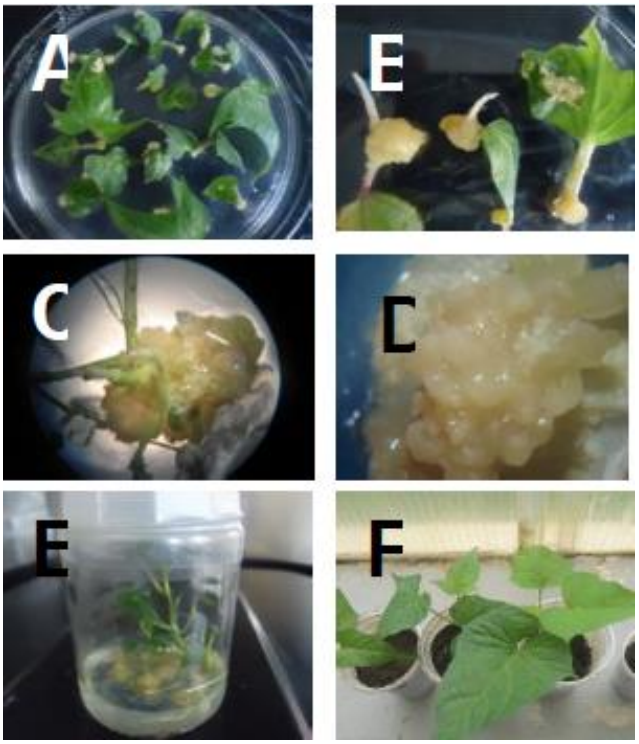

Figure 2. Induction of embryo-like structures and regeneration of shoots and roots in African sweet potato cultivars. **A**. Induction of callus from the base of petioles attached to leaves. **B.** Root regeneration from callus before development of embryo-like structures. **C.** regeneration of multiple roots from callus after 5 weeks on callus induction medium. **D.** Development of embryo-like structures after placement of embryogenic callus on ABA-supplemented medium. **E.** Regeneration of shoots from callus of cv. Jonathan after transfer to plant regeneration medium. **F.** Survival of plants after transfer from in vitro cultures to soil in screen house.

a highly significant ($P < 0.001$) effect on the ability to regenerate roots (Table 2).

DISCUSSION

Sweet potato has for long been considered recalcitrant to somatic embryogenesis (Yang et al., 2011). Most of the reported protocols are cultivar specific, and have low regeneration frequencies which are also difficult to reproduce (Santa-Maria et al., 2009; Yang et al., 2011; Yu et al., 2007). In the present study, the auxin 2, 4-D was used for callus induction and embryo-like structures was achieved in sixteen of the investigated twenty African cultivars. These cultivars have not been reported to respond to somatic embryogenesis in previous publications. Luo et al. (2006) reported that African cultivars were difficult to regenerate, but did not name the cultivars tested or their geographical location.

The induction of somatic embryogenesis with 2, 4-D appears to be preferable in view of existing literature (Yang et al., 2011). Liu et al. (2001) induced embryo-like structures from shoot apices of fifteen cultivars from China and Japan on medium supplemented with 9.05 mM 2, 4-D. The embryogenic response of these cultivars was low and so variable ranging from 6.7 to 85.2% (Liu et al., 2001). Similarly, Sihachakr et al. (1997) found large differences and low frequency of embryo-like structures among ten sweet potato cultivars exposed to auxin (10 μM 2, 4-D) treatment. Frequencies ranged from 0 to 17% response when using lateral buds as explants (Sihachakr et al., 1997). The frequency of embryo-like structures in the current study ranged from no response at all to 25% depending on cultivar (Table 2). In contrast to somatic embryogenesis protocols, most auxin-cytokinin shoot organogenesis protocols have replaced 2, 4-D with other auxins in order to avoid the callus stage and achieve rapid regeneration (Luo et al., 2006, Gong et al., 2005; Santa-Maria et al., 2009).

Although, somatic embryogenesis is a complicated process to manage than shoot organogenesis (Luo et al., 2006; Newell et al., 1995), the regeneration of sweet potato through somatic embryogenesis is still a preferred option than shoot organogenesis (Yu et al., 2007). This is mainly because somatic embryogenesis leads to high efficiency of selection of transgenic plants on medium and avoids the regeneration of chimeric plants after genetic transformation (Song et al., 2004; Yang et al., 2011). Recently, there have been some reports of applying somatic embryogenesis using cell suspensions (Yu et al., 2007; Zang et al., 2009). However, other reports suggest that the use of liquid cultures is extremely complicated and depends on availability of cell suspension cultures (Song et al., 2004; Zhai and Liu, 2003). By opting for solid medium in this study, it was possible to deploy explants which are easy to access and are readily available as noted in previous reports (Cipriani et al., 1999; Kreuze et al., 2008; Song et al., 2004). The use of solid medium for somatic embryogenesis has allowed many researchers to use various plant organs as explants for example, leaf discs (Newell et al., 1995), stem internodes (Song et al., 2004), root explants (Liu and Cantliffe, 1984), root discs (Newell et al., 1995) and lateral buds (Cavalcante Alves et al., 1994).

The regeneration of roots in this work could be useful for the initiation of root cultures, which could serve as a source of tissue for regeneration of plants or hairy root cultures (Jones et al., 2007; Dodds et al., 1991). However, it remains difficult to explain this type of morphogenesis which was evident as early as three weeks after culture initiation on 2, 4-D-supplemented medium. It is likely that the explants of the cultivars had high levels of endogenous auxins. If this is the case then the exogeneous auxin 2, 4-D only accelerated the root regeneration process (Becerra et al., 2004; George et al., 2008). Many factors have been implicated in the unpredictable responses in culture, including variations in the developmental and physiological stage of *in vitro*

Table 2. Embryogenic callus formation from 'whole leaf' explants of 21 sweet potato cultivars and regeneration of roots and shoots.

Cultivar	Explants tested (No.)	Explants inducing callus		Explants Inducing embryogenic callus		Explants regenerating roots		Explants Regenerating shoots	
		No.	%	No.	%	No.	%	No.	%
Naspot 1	13.3 ±0.3	10±1.5	75.0±10.4	0±0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Dimbuka	13.7 ±0.7	12.3±0.7	90.2±4.8	1.3±0.7	9.8±4.8^{bcde}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Semanda	14.3 ±0.3	12.3±1.8	86.0±10.9	0.3±0.3	2.3±2.2^{ab}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
New Kawogo	13.3 ±1.2	12±1.5	90.0±3.8	3.0±0.6	22.5±6.7^{ef}	1.0 ± 0.6	7.5±3.9^{b}	0.0 ± 0.0	0.0 ± 0.0^{a}
Bwanjule	14 ±0.6	12±1.0	85.7±4.3	1.3±0.9	9.5±6.8^{bcde}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Namusoga	14.7 ±0.3	11.3±0.3	77.3±2.0	0±0.0	0.0±0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Jamada	13.7 ±0.9	10.7±1.5	78.0±5.8	0.3±0.3	2.4±2.8^{ab}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Magabali	14.7 ±0.3	12.7±1.3	86.4±10.2	3.7±0.3	25±1.7^{f}	2.0 ± 0.6	13.6±3.7^{cd}	0.0 ± 0.0	0.0 ± 0.0^{a}
Kyebandula	14 ±0.6	12.3±0.3	88.1±2.1	2.7±0.3	19.0±3.0^{def}	1.3±0.9	9.5 ± 5.9^{bc}	0.0 ± 0.0	0.0 ± 0.0^{a}
Jonathan	14 ± 0.6	11±0.6	78.6±7.4	2.3±0.3	16.7±1.8^{cdef}	3.0±0.6	21.4 ± 3.9^{d}	1.3 ± 0.9	9.5 ± 6.8^{b}
Kakamega	14.7±0.3	11.3±2,2	77.3±13.5	0.0 ± 0.0	0.0±0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Araka red	13.7±0.3	10.7±1.3	78±10.8	0.7±0.7	4.9±4.8^{ab}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Naspot 8	14.3±0.7	12.0±0.6	83.7±2.0	0.0±0.0	0.0±0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Munyeela	13.7±0.9	11.0±1.0	80.5±7.7	0.7±0.7	4.9±4.4^{ab}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Kisakyamaria	13.3±0.9	9.7±0.9	72.5±3.0	0.0 ±0.0	0.0±0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Silk omupya	13.7±1.3	9.7±1.7	70.7±9.7	1.3±0.7	9.8±5.4^{bcdef}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Luwero Silk	14.3±0.3	10.3±0.7	72.1±4.2	0.0 ±0.0	0.0±0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Kigaire	13.7±0.9	9.7±0.7	70.7±5.7	0.7±0.3	4.9±2.5^{abc}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Nyidoyamulalo	14.3±0.3	10.7±0.7	74.4±5.7	0.3±0.3	2.3±2.4^{ab}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
Luwero 2 (uknown)	14.7±0.3	13.3±0.7	90.9±2.5	1.3±0.9	9.1±5.9^{abcd}	1.0 ± 0.6	6.8 ± 4.1^{b}	0.0 ± 0.0	0.0 ± 0.0^{a}
Luwero (uknown)	14.3±0.7	10.0±1.0	69.8±3.9	1.0±0.6	7±4.5^{abcd}	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}

F-test	Explants inducing callus (%)	Explants inducing embryogenic callus (%)	Explants regenerating roots (%)	Explants regenerating shoots (%)
Cultivar	ns	< 0.001	< 0.001	< 0.001

Data represent means ± standard errors for three replicates (10 to 15 explants each). Values followed by the same letter in each column are not significantly (ns) different at the P ≤ 0.05 level (LSD test).

plants (Triqui et al., 2007). Previous reports indicate that ABA is required for induction, maturation and synchronization of somatic embryos leading to elongation into shoots (Anwar et al., 2010; Song et al., 2004; Triqui et al., 2007). However, in the current study, two Ugandan cultivars, Magabali and Kyebandula that induced more embryo-like structures than the embryogenic callus reported for the USA cultivar, Jonathan, did not regenerate shoots. The failure of ABA to induce somatic embryos or promote development of preformed somatic embryos in the sixteen African cultivars that successfully induced embryo-like structures could be attributed to cultivar differences in the response to ABA or the sustained effect of 2,4-D as reported previously (Becerra et al., 2004; George et al., 2008; Santa-Maria et al., 2009).

In conclusion, this study has identified up to sixteen African sweet potato cultivars which were able to induce embryo-like structures on medium supplemented with the potent auxin 2,4-D (0.2 mg/L). The embryo-like structures induced in this study could be useful for the initiation of embryogenic cell suspensions which are not readily available for transformation of most sweet potato cultivars (Yang et al., 2011). The method

for *de novo* regeneration of roots may be applicable in the regeneration of plants or hairy root cultures. A possibility exists that the use of other types of plant organs as explants and various concentrations of 2, 4-D or other auxins could improve somatic embryogenesis in the cultivars investigated. This view is now being pursued as a follow-up study. The final goal of this work is to use the somatic embryogenesis protocol for *Agrobacterium*-mediated transformation in order to improve sweet potato traits.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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