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Effect of genotype, gelling agent, and auxin on the induction of somatic embryogenesis in sweet potato (*Ipomoea batatas* Lam.)

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Abstract

Lateral buds of six cultivars of sweet potato were induced to form embryogenic callus in a culture medium solidified with two types of gelling agents, Agar or Gelrite, and supplemented with various concentrations of auxins, 2,4-D, 2,4,5-T and Picloram. Of the six cultivars screened, only three gave an embryogenic response. Best results with an average of 3.53% embryogenic response were obtained with the medium solidified with Agar, while in Gelrite only 0.45% of lateral buds gave rise to embryogenic callus. The interaction between the genotype and auxins was highly significant; particularly the optimal response was obtained with cv. Zho and 865 yielding 10.7 and 14.7% somatic embryogenesis, respectively, in the medium containing 2,4,5-T or Picloram. The plant conversion was dramatically improved by subculture of the embryogenic callus on the medium with the combination of 1 μM 2,4-D and 1 μM Kinetin or 5 μM ABA alone before transfer of mature embryos onto hormone-free medium. The embryogenic callus of sweet potato and its sustained ability to further regenerate plants have regularly been maintained for several years by frequent subculture in 5 μM 2,4,5-T or the combination of 10 μM 2,4-D and 1 μM BAP or kinetin. The embryo-derived plants seemed apparently genetically stable and similar to the hexaploid parental plants, based on morphological analysis and their ploidy level determined by using flow cytometry. ***To cite this article: Z. Triqui et al., C. R. Biologies 331 (2008).***

Résumé

Effet du génotype, de l'agent gélifiant et de l'auxine sur l'induction de l'embryogenèse somatique chez la patate douce (Ipomoea batatas Lam.). Des bourgeons axillaires de six cultivars de patate douce ont été ensemencés sur des milieux d'induction de cals embryogènes solidifiés par deux types d'agents gélifiants, qui sont l'agar et la gelrite, et additionnés de 2,4-D, 2,4,5-T ou Piclorame à différentes concentrations. Parmi les six cultivars testés, seuls trois ont donné une réponse embryogène. Les meilleurs résultats, avec une moyenne de 3,53%, ont été obtenus avec l'agar, alors qu'avec la gelrite, seuls 0,45% des bourgeons axillaires ensemencés ont donné une réponse embryogène. L'interaction entre auxine et génotype s'est montrée hautement significative.

Abbreviations: ANOVA, analysis of variance; ABA, abscisic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indol-3-acetic acid; MS, Murashige and Skoog basal medium; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid.

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L'optimum des réponses embryogènes a été obtenu avec les cv. Zho et 865, qui ont produit respectivement un pourcentage de cals embryogènes de 10,7 et 14,7%, en présence de 2,4,5T et Piclorame. La conversion des plantes a été fortement améliorée par le repiquage des cals embryogènes sur un milieu comprenant une combinaison de 2,4-D et de Kinétine ou de BAP à 1 μM chacune ou l'ABA seul à 5 μM avant de transférer les embryons mûrs sur un milieu sans régulateurs de croissance. La capacité embryogène des cals des variétés Zho et 865 a été maintenue pendant plusieurs années par repiquage régulier sur un milieu comprenant 5 μM de 2,4,5-T ou une combinaison de 10 μM 2,4-D et 1 μM de BAP. Les plants issus des embryons somatiques du cv. Zho se sont montrés génétiquement stables en se basant sur la morphologie et sur le niveau de ploïdie mesuré par la cytométrie en flux. ***Pour citer cet article : Z. Triqui et al., C. R. Biologies 331 (2008).***

Keywords: Auxin; Flow cytometry; Gelling agent; *Ipomoea batatas*; Somatic embryogenesis; Sweet potato

Mots-clés : Agent gélifiant ; Auxine ; Cytométrie en flux ; Embryogenèse somatique ; *Ipomoea batatas* ; Patate douce

1. Introduction

Sweet potato (*Ipomoea batatas* Lam.) is a tuber-bearing species and represents an economically important crop in tropical, subtropical and warm temperate regions [1]. The world production of sweet potato was estimated at 129.4 Mt in 2005, of which more than 88% were from Asian countries, particularly China, with 107.1 Mt [2]. The storage roots contain a high amount of starch, which is as high as 30% of fresh weight for some cultivars. They are used as staple food, raw material for alcohol production, and animal feed. Stems and foliage are also used as forage.

Although progress has been made in the improvement of sweet potato by using conventional breeding methods [1], the selection process is time-consuming and requires a high number of individuals and improved breeding systems, because of the hexaploid status of this species ($2n = 6x = 96$ chromosomes) [3]. Moreover, breeding efforts have been seriously limited by difficulties in sexual crosses, mainly due to incompatibility and sterility within species of sweet potato [4], as well as to specific physiological requirements for flowering [5]. Therefore, biotechnology has been developed to complement and supplement the classical methods in breeding programs for efficient improvement of this crop. Despite the economic importance of sweet potato, biotechnological applications are still in their infancy. Only little work has been achieved, particularly the exploitation of somaclonal variation [1], somatic hybridization [6] and genetic transformation [7]. One of the main difficulties is the control of plant regeneration, for which sweet potato is considered a recalcitrant species [8]. Among various systems of plant regeneration, somatic embryogenesis is highly desired, as the process regularly provides high multiplication rates and can effectively be maintained for a long time. Genotype has been shown to be a limiting factor in induction of somatic embryogenesis, as many cultivars of sweet potato gave low or no embryogenic responses at all [9–11]. Rapid and repetitive plant regeneration via somatic embryogenesis was demonstrated in only one genotype of sweet potato, PI318846-3 [12]. The type of auxin used was also found critical for successful induction of somatic embryogenesis [13,14]. Moreover, the gelling agent, which is an often-neglected parameter in the protocols for plant regeneration, can interfere with the ability of the medium to induce the desired response, such as induction of somatic embryogenesis or shoot formation [15,16]. The two gelling agents, which have so far most commonly been used to solidify the culture medium, are Agar or its purified derivative, Agarose, extracted from red algae such as *Gracilaria*, *Gelidium* or *Chondrus*, and gellan gum, also known as Gelrite or Phytagel, an extracellular polysaccharide produced by the bacterium *Sphingomonas elodea*. Although several factors have been shown to be involved in induction of somatic embryogenesis and plant regeneration, in most experiments their effects were examined separately, resulting in missing crucial information on the possible interaction between such parameters in embryogenic response. Besides, the occurrence of a callus phase during tissue culture, particularly the culture of sweet potato protoplasts, may result in the generation of variants with morphological and physiological abnormalities, reflecting the genetic instability [8,17].

In this study, the investigation was extended to examine the effect of genotype, gelling agent and auxin, as well as of their interaction, on the induction of somatic embryogenesis in sweet potato. We compared the effects of two gelling agents, Agar and Gelrite, combined with those of three auxins, 2,4,5-T, 2,4-D and Picloram, used at two concentrations on the induction of somatic embryogenesis in six cultivars of sweet potato. Moreover, the genetic stability of embryo-derived plants was

also examined through the determination of their ploidy levels by using flow cytometry.

2. Materials and methods

2.1. Plant materials

Six clones of sweet potato (*Ipomoea batatas* Lam.; Convolvulaceae) were used. Five clones, cultivars Quangshu, Zho, 953, 865 and 90, were obtained from the Xuzhou Sweet Potato Research Centre, China, and one clone, cv. Duclos 11, from the 'Institut national de la recherche agronomique' (INRA) of the French West Indies. The plant materials were maintained and multiplied in vitro by subculture of leafy node-cuttings, at intervals of six weeks, on basal MS medium [18], supplemented with vitamins [19], 20 $g\,L^{-1}$ sucrose, 0.5 $mg\,L^{-1}$ IAA and solidified with 7 $g\,L^{-1}$ agar [17]. The cultures were kept in a growth chamber at $27 \pm 2\,^{\circ}C$ with 16 $h\,day^{-1}$ at 62 $\mu M\,m^{-2}\,s^{-1}$, and 60% humidity.

2.2. Induction of somatic embryogenesis and plant regeneration

For induction of somatic embryogenesis, lateral buds with 2 to 4 leaf primordia were taken from the 10 to 12 nodes from apices of 7–8-week-old plants with a sterile hypodermic needle and under a binocular microscope. About 10 buds were placed on the solidified induction medium contained in a 10×60-mm Petri dish. The medium was composed of basal MS medium, vitamins [19], 30 $g\,L^{-1}$ sucrose and supplemented with one of the three different auxins, 2,4-D, 2,4,5-T used at two concentrations, 5 and 10 μM, and Picloram at only 10 μM. The growth regulators were added to the medium before autoclaving. The medium was solidified with either 7 $g\,L^{-1}$ Agar (Bacto Agar – DIFCO) or 3 $g\,L^{-1}$ Gelrite. The pH was adjusted to 5.8. The induction was carried out in darkness at $27 \pm 2\,^{\circ}C$. Embryogenic calli were then divided into fragments of 2–3-mm diameter, and subcultured on the multiplication medium with the same composition as for induction. Further development into embryos and their conversion into plants required the transfer of embryogenic calli onto either hormone-free medium or supplemented with combination of growth regulators, particularly ABA (Sigma) for embryo maturation. For callus multiplication and plant regeneration, the cultures were kept in the growth chamber with the same conditions of temperature and illumination as for plant micropropagation, i.e. $27 \pm 2\,^{\circ}C$ with 16 $h\,day^{-1}$ at 62 $\mu M\,m^{-2}\,s^{-1}$, and 60% humidity.

2.3. Determination of the ploidy level by using flow cytometry

Among hundreds of plants derived from somatic embryos of cv. Zho, a sample of 50 clones was randomly selected and multiplied for the study of the genetic stability. Flow cytometry was used to quantify DNA for determination of the ploidy level, as described by Fock et al. [20]. Briefly, about 1 cm^2 of leaf material from in vitro plants was chopped with a razor blade in 1 ml of a buffer solution containing CPW salts [21], 0.5 M mannitol, 0.25% (w/v) PEG, 0.5% (w/v) Triton X-100, 0.25% (v/v) mercaptoethanol at pH 6.5–7.0. Crude samples were filtered through a 40-μm mesh nylon and stained with 4,6 diaminido-2-phenylindol (DAPI, 5 $\mu g\,ml^{-1}$). DNA analysis was performed on a PARTEC CA II flow cytometer (Chemunex, Maison-Alfort, France) equipped with a 100-W mercury lamp (type HBO). Blue fluorescence at 455 nm was recorded as a function of the DNA content. Because of their exceptional stability and regularity, murine resting splenic B lymphocytes were used to calibrate the fluorescence scale. The external references of the hexaploid status was provided by the use of the hexaploid (6X) parental plants of sweet potato ($2n = 6x = 96$ chromosomes) [3]. The DNA distribution was analysed, by using DPAC software, on histograms generated from at least 10,000 nuclei.

2.4. Statistical analysis

Each experiment of induction of somatic embryogenesis involving the combination of factors was repeated at least six times, using a total of nearly 4000 lateral buds. The number and percentage of embryogenic responses were collected 10 weeks after induction. The data, expressed in frequency (x), were transformed into $\arcsin\sqrt{x}$ (angular transformation) and the frequency zero into $1/(4n)$ [22], n being the number of explants. The transformed variates were subjected to statistical analysis, using a fixed model of analysis of variance (ANOVA) with one or two criteria of cross classification. Duncan's multiple-range test [23] was used for means separation.

3. Results

3.1. Induction of the embryogenic callus

The lateral buds (Fig. 1A) swelled slightly after two weeks of culture in the induction medium. They lost their compact consistency and 1.5–10.7% of cultured

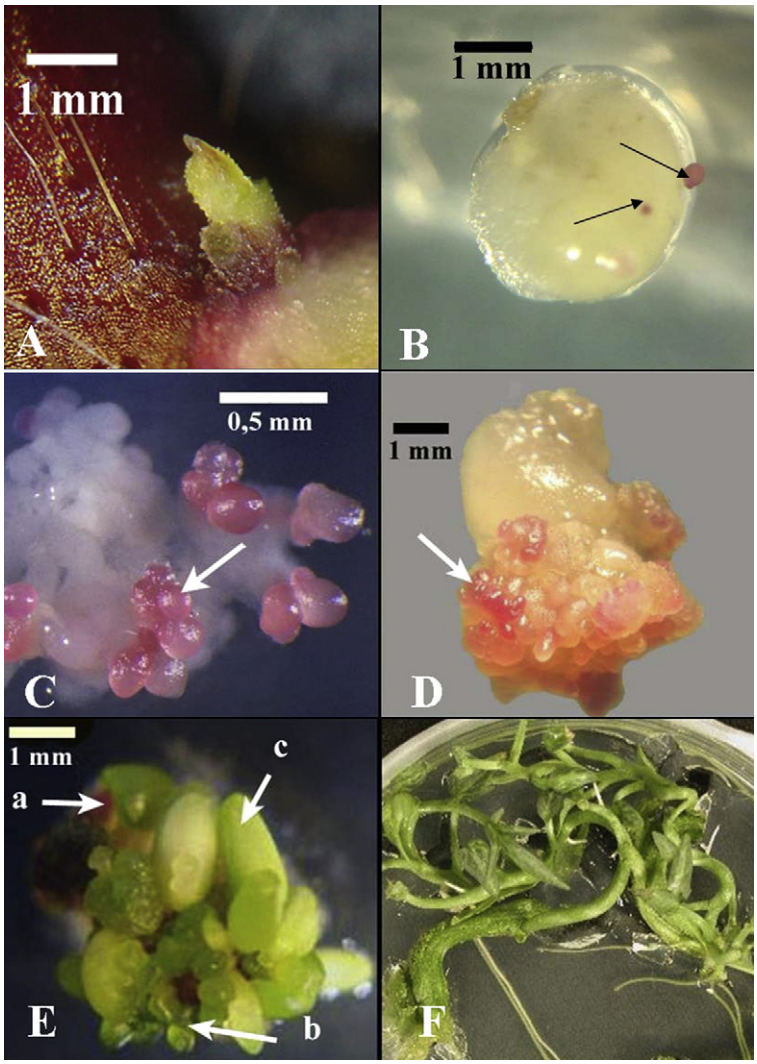

Fig. 1. (**A**) Initial explant at time of culture. (**B**) Reddish embryonic callus (arrow) seen through milky mucilaginous callus. (**C**) Embryonic red structures (arrows) that have emerged from milky callus. (**D**) Reddish embryonic nodular callus (arrow) on a cellular mucilaginous mass. (**E**) Callus with embryos at various stages (**a**, red globular embryos; **b**, green heart shaped embryos; **c**, green cotyledonary embryos). (**F**) Somatic embryos developing into plants. For interpretation of colours, see the web version of this article.

buds, depending on the medium and variety, rise to a mucilaginous milky mass in which compact embryogenic calli developed from some explants (Fig. 1B), while most of them became either a small callus or friable fast-growing non-embryogenic callus. Generally, the small callus rapidly turned brown and died within a few days. The fast-growing friable callus was white to brown in colour, and composed of large translucent cells. It never gave rise to embryogenic structures. The compact embryogenic callus was yellowish or white and comprised pink or red areas, due to the presence of anthocyanin, depending on the genotype, particularly cv. Zho (Fig. 1C and D). It was composed of very tightly packed isodiametric cells. In the regeneration medium, embryos appeared; they were observed at different stages (Fig. 1E). These bipolar structures developed root before activation of stem meristem apex, leading to complete plants (Fig. 1F).

3.2. *Effect of gelling agent*

The effect of two types of gelling agent, Agar and Gelrite, was assessed for induction of embryogenic response in six cultivars of sweet potato (Table 1). The ANOVA of the embryogenic response (%) showed very highly significant effects of the gelling agent and geno-

Table 1
Percentage of embryogenic response of six cultivars of sweet potato after 10 weeks of culture in the induction medium supplemented with two concentrations (5 and 10 μM) of 2,4,5-T and solidified with either Agar or Gelrite. The values followed by the same letter are not significantly different at $P = 0.05$

Genotype	Agar		Gelrite		Means
	5 μM 2,4,5-T	10 μM 2,4,5-T	5 μM 2,4,5-T	10 μM 2,4,5-T	
Zho	10.70ab	10.07ab	0.00d	1.78cd	5.64
Quangshu	0.00d	0.00d	0.00d	0.00d	0.00
953	0.00d	0.00d	0.00d	0.00d	0.00
865	14.68a	6.92bc	0.00d	2.14d	5.94
90	0.00d	0.00d	0.00d	1.46d	0.37
Duclos 11	0.00d	0.00d	0.00d	0.00d	0.00d
Means	4.23	2.83	0.00	0.90	1.99

Table 2
Effect of auxins (2,4-D, 2,4,5-T and Picloram) on the induction of somatic embryogenesis in six cultivars of sweet potato. The embryogenic response (%) was observed after 10 weeks of culture in the induction medium solidified with 7 g L^{-1} agar. The values followed by the same letter are not significantly different at $P = 0.05$

Genotype	Picloram	2,4,5-T		2,4-D		Means
	10 μM	5 μM	10 μM	5 μM	10 μM	
Zho	10.60ab	10.70ab	10.07ab	0.69cdef	0.00cdef	6.41
Quangshu	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00
953	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00
865	6.59abc	14.70a	6.92abcd	6.37bcde	0.00cdef	6.92
90	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00
Duclos 11	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00cdef	0.00
Means	2.87	4.23	2.83	1.18	0.00	2.22

type. Irrespective of the genotype, the medium solidified with Agar gave the best embryogenic response with 3.53%, while very few lateral buds in Gelrite were induced to form embryogenic callus, yielding only 0.45%. Of the six cultivars screened, only three, i.e. Zho, 865 and 90, gave rise to an embryogenic response (Table 1). The best embryogenic response was obtained from the cultivars Zho and 865, particularly in the medium solidified with Agar and containing a low level of 2,4,5-T (5 μM), giving 10.70 and 14.68% of embryogenic callus, respectively. Despite the non-significance of the interaction effect between the gelling agent and genotype, only 1.46% of lateral buds of cultivar 90 were induced to form the embryogenic callus in the medium containing Gelrite and a high level of 2,4,5-T (10 μM). This low frequency of embryogenic response was not significantly different from zero (Table 1).

3.3. Effect of auxin and genotype

Comparison was made between three types of auxin, 2,4-D, 2,4,5-T used at 5 and 10 μM, and Picloram at 10 μM for the assessment of the embryogenic response in six sweet potato cultivars cultured on a medium solidified with Agar (Table 2). The ANOVA of the frequency of embryogenic response showed very highly significant effects of the type of auxin and the genotype, as well as their interaction (Table 2). This indicates that the effect of the auxin type on the embryogenic response varied with the genotype of sweet potato. Surprisingly, only two cultivars, Zho and 856, were induced to form few embryogenic calli in medium with a low level of 2.4-D (5 μM), while a high level of 2,4-D (10 μM) induced no embryogenic response at all (Table 2). On the contrary, best results were obtained in cv. Zho with 2,4,5-T or Picloram, yielding a mean of 10.33% of embryogenic response whatever the concentration used. The genotype 865 gave 14.7% of embryogenic response in 5 μM 2,4,5-T and only 6.59% in 10 μM Picloram (Table 2).

3.4. Maintenance of embryogenic callus and plant regeneration

The compact embryogenic callus was multiplied by regular subcultures in the fresh medium used for embryogenic induction at intervals of 2 months. The cultures were kept in darkness. During successive subcul-

Table 3
Estimation of the ploidy level of a sample of embryo-derived clones of sweet potato, cv. Zho, by using flow cytometry. The mean value of the dominant peak of a sample of 50 clones analysed was estimated at 82.5 ± 0.6, the value of the plant control, cv. Zho, being at the channel 83 ± 0.56

Embryo-derived clones	Control line cv. Zho	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Dominant peaks (Go/G1) nuclei No. channel	83	85	82	81	80	82	84	80	85	80	81
Coefficient of variation (%)	5.6	6.2	4.3	5.5	6.3	4.5	6.5	5.5	5.3	6.1	5.3
Ploidy level (*X*)	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*	6*X*

tures, the embryogenic callus grew up and developed into a new embryogenic callus. Besides, it also formed a mucilaginous milky mass from which emerged compact embryogenic callus. Nevertheless, after several subcultures, some sectors of compact embryogenic callus became necrosed or friable and non-embryogenic. The non-embryogenic callus had to be carefully removed before each subculture, as it grew twice as fast as the embryogenic callus and rapidly invaded all the cultures. Moreover, it could never be induced to form embryogenic callus again, thus losing the ability to regenerate plants. Better proliferation of embryogenic than of non-embryogenic callus could selectively be enhanced on the multiplication medium containing 5 μM 2,4,5-T or the combination of 10 μM 2,4-D and 1 μM BAP or kinetin. By applying this procedure, embryogenic calli of sweet potato, with sustained ability to further regenerate plants, have regularly been maintained for several years.

For further differentiation of embryos, the compact embryogenic calli subcultured on the induction or multiplication medium were illuminated as for plant propagation. After four weeks in the light, embryos at globular stage were developed, but their further development into advanced stages was achieved upon their transfer to hormone-free medium. However, the maturation of embryos and particularly their conversion to plants was dramatically improved by subculture on medium with the combination of 1 μM 2,4-D and 1 μM Kinetin before transfer of mature embryos onto hormone-free medium for further plant development. ABA alone was also tested at 2.5, 5, 10 and 15 μM concentrations (data not shown), and the optimal maturation of embryos was obtained with 5 μM. Those treatments have resulted in successful regeneration of 5–10 plants per cluster of embryogenic callus within less than two months. The embryo-derived plants displayed apparently normal morphology. They rooted well and grew vigorously when subcultured on basal MS medium supplemented with 0.5 mg L^{-1} IAA for multiplication.

3.5. Determination of the ploidy level

The ploidy level of regenerated plants was determined by comparing the position of dominant peaks corresponding to nuclei at G0–G1 phases of the cell cycle, between embryo-derived plants and parental lines. The analysis showed that the dominant peak of the hexaploid parental line, cv. Zho, was located at channel 83, and that of the sample of 50 embryo-derived clones, which had been examined, ranged from channels 80 to 85, giving a mean value of 82.5 ± 0.6 (Table 3). This mean value was practically identical to that of the control plants as the values of the coefficient of variation were relatively high, ranging from 4.3 to 6.5%. Consequently, the ploidy level of embryo-derived plants could be considered identical to that of the original hexaploid (6x) plants of sweet potato.

4. Discussion and conclusion

In this study, somatic embryogenesis was regularly induced in three cultivars of sweet potato, cv. Zho, 90 and 865. A very compact embryogenic callus developed from a mucilaginous milky mass. The resulting embryogenic callus with its potential of plant conversion has constantly been maintained for more than two years by subculture in a medium supplemented with 5 μM 2,4,5-T or a combination of 10 μM 2,4-D and 1 μM BAP or kinetin. The gelling agent and combination of growth regulators were shown to have a significant effect on the induction of somatic embryogenesis in sweet potato. So far, in tissue cultures, the gelling agent has generally been considered only a solid support, preventing plant explants from immersion and suffocation. The use of solidified medium reduces the diffusion phenomenon, thus limiting the movement of nutrient elements [24]. Moreover, the growth of cultured plant explants may be affected by inhibitive substances released by necrosed plant tissues, and which concentrate near the explants [25]. Those effects depend on the

type and concentration of the gelling agent used. Gelrite was reported to provide a better availability of water [26,27]. Similarly, inhibitive molecules, such as phenols produced by plant explants, are fluently diffused in the medium solidified with Gelrite [28]. Moreover, this gelling agent contains a higher level of Ca, K, Mg and Fe ions, compared to the medium solidified with Agar, which has three times as many Na ions [29–31]. In this study, a better induction of somatic embryogenesis in sweet potato was obtained by using Agar as the gelling agent. In liquid medium, somatic embryogenesis was completely inhibited (data not shown). This may also explain the lesser induction in Gelrite, where water is more available, despite the high concentration of this gelling agent (3 $g\,L^{-1}$) used in this work.

In this study, the interaction between the genotype and the combination of growth regulators was found significant. Induction of somatic embryogenesis was successful in a medium with a low level of 2,4-D for two cultivars, Zho and 865, and with a high level of 2,4-D for cv. 90. The best results were obtained with 2,4,5-T and Picloram, as already observed in previous studies [13,14]. In this work, cultivars Duclos 11, Guangshu and 953 did not give any embryogenic response whatever the treatments used, whereas cv. Guangshu and 953 were previously reported to form embryogenic callus [10]. The absence of embryogenic response in those genotypes may be due to a variation in the developmental and physiological stage of vitroplants, affecting the cultural behaviour of lateral buds used for induction. In fact, it was shown that the embryogenic response in sweet potato highly depended on the size of lateral buds incubated. Only buds with a size of 0.5–1 mm were suitable for embryogenic induction [14]. Endogenous factors, such as the level of growth regulators within plant explants, together with the interaction between endogenous and exogenous hormones may also influence the embryogenic response [32]. Moreover, the ability to develop somatic embryogenesis was also shown to be under genetic control [33], and particularly in sweet potato, the embryogenic response depended on the genotype [10,14,34]. In this study, the absence of embryogenic response was confirmed for cv. Duclos 11, as already shown in a previous work [10]. Similarly, cv. Jewel, which was used in several breeding programs of sweet potato, was also reported to be unable to give an embryogenic response [11,35].

The presence of abscisic acid was found crucial for the maturation of sweet potato embryos [12,13,34], as the plant conversion obtained in this work was dramatically improved, resulting in the development of morphologically normal plants. The embryo-derived plants seemed genetically stable and similar to the hexaploid parental plants, based on morphological analysis, and their ploidy level determined by using flow cytometry [3,8,36]. The relative genetic stability of the regenerated plants may be explained by the meristematic organisation of the initial explants used for culture and the brief period of callus phase. Further analysis and evaluation, particularly in the field conditions, are in progress to confirm the conformity and genetic stability of those plants regenerated through somatic embryogenesis in sweet potato.

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