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Assessment of somaclonal variation in somatic embryo-derived plants of yacon [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Robinson] using inter simple sequence repeat analysis and flow cytometry

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ABSTRACT

Background: Yacon (*Smallanthus sonchifolius*) is a root crop native to the Andean region. Low sexual reproductive capacity is a major constraint facing the genetic breeding of this crop. Biotechnological techniques offer alternative ways to widen genetic variability. We investigated somaclonal variation in regenerants of yacon derived from *in vitro* somatic embryogenesis using simple sequence repeat (ISSR) analysis and flow cytometry.
Results: Twenty tested ISSR primers provided a total of 7848 bands in 60 *in vitro* regenerants and control plant. The number of bands for each primer varied from 3 to 10, and an average of 6.95 bands was obtained per ISSR primer. Eight primers were polymorphic and generated 10 polymorphic bands with 7.19% mean polymorphism. ISSR analysis revealed genetic variability in 6 plants under study. These regenerants had Jaccard's distances 0.104, 0.020, 0.040, 0.106, 0.163 and 0.040. Flow cytometric analysis did not reveal changes of relative nuclear DNA content in regenerants suggesting that the plants obtained *via* somatic embryogenesis had maintained stable octoploid levels.
Conclusions: Our findings show that indirect somatic embryogenesis could be used in yacon improvement to widen genetic variability, especially when low sexual reproductive capacity hinders classical ways of breeding.

1. Introduction

Yacon [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Robinson] from the *Asteraceae* family is a perennial herb native to the Andean region and is known to have been cultivated since pre-Inca times. Yacon storage roots are a particularly abundant source of fructooligosaccharides, consisting of a series of inulin type β (2 → 1) fructans, which have a positive effect on human health [1,2]. The aerial part of yacon shows strong antimicrobial activity [3], and the whole plant contains phenolic compounds with antioxidative activity [4].

In different landraces, there appears to be significant variation in morphological traits [5], as well as in antioxidative activity and fructooligosaccharide content [6]. However, genetic divergence among landraces, as revealed by molecular markers, is very low. This can be explained because of high levels of vegetative propagation and long term selection for desired agronomic traits in yacon [7]. Moreover, low germination of pollen, few achenes with seeds and low seed viability cause sexual reproduction of yacon to be very rare [8].

Low sexual reproductive capacity is also a major constraint facing genetic breeding of this crop [8]. Biotechnological techniques, such as induction and selection of somaclonal variation, *in vitro* chromosome doubling and genetic transformation offer alternative ways to widen genetic variability of the crop [9]. Numerous studies have been reported on *in vitro* propagation of *S. sonchifolius via* shoot tips, axillary buds or somatic embryos [10,11,12]. Nevertheless, in these studies, genetic fidelity of regenerated plants had not been assessed, though especially the presence of a disorganized growth phase in tissue culture is considered as one of the factors that may cause somaclonal variation [13]. Although occurrence of uncontrolled variation during the culture process is mostly an unexpected and undesired phenomenon [14], it can also be an important tool for plant breeding *via* generation of new varieties with useful agronomic traits [13].

Somaclonal variation resulting from point mutations or the activation of mobile elements can be detected by DNA-based marker systems, such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter

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simple sequence repeat (ISSR) [13,15,16]. The low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism are the major limitations of the first three methods. ISSR marker is a technique that overcomes most of these limitations. Moreover, it is a very simple, fast, cost-effective, highly polymorphic and reliable method [17]. It requires only a small quantity of a DNA sample and it does not need any prior sequence information to design the primer [18], thus it is suitable for the assessment of the genetic variation among *in vitro* regenerants.

The use of flow cytometry for detection of DNA content is also a very convenient method to reveal somaclonal variation in regenerated plants, as *in vitro* cultivation is also being associated with ploidy level changes [13,19]. Especially *in vitro* long-term cultivation, plant regeneration *via* indirect morphogenesis and preexistence of ploidy variation in explant of polysomatic species may result in ploidy instability of *in vitro* regenerated plants [20,21]. Flow cytometry has several advantages compared to other methods; unlike chromosome countings, it is a rapid method for ploidy testing since it allows the examination of large numbers of cells as well as different types of tissues and cell layers [22].

In the present study, we examined somaclonal variation in plants regenerated *via* somatic embryogenesis, where somatic embryos were initiated using different growth regulators at various levels. Two methods were employed, ISSR markers and flow cytometry. To our knowledge this is the first report in *S. sonchifolius* on the assessment of somaclonal variation in regenerated plantlets from indirect somatic embryogenesis.

2. Materials and methods

2.1. Plant material

Experiments were carried out on an octoploid clone of *S. sonchifolius* ($2n = 8x = 58$), originated in Ecuador. *In vitro* plants were cultured on MS medium [23] containing 1 mg l^{-1} thiamine, 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose and 8 g l^{-1} agar, at pH 5.7. Cultures were maintained for 30 d at 25/23°C under a 16/8 h light/dark regime with 36 μmol m^{-2} s^{-1} cool white fluorescent light. Young petioles were excised from the plants. The petioles were cut into ca. 5 mm long segments and placed with the abaxial surface toward the medium, five pieces to each flask. These segments were used as an initial culture for somatic embryogenesis experiments.

2.2. Regeneration of plants via somatic embryogenesis

Petiole segments were cultivated in Erlenmeyer flasks (100 ml) with 25 ml of MS medium containing 30 g l^{-1} sucrose, 1 mg l^{-1} thiamine, 100 mg l^{-1} *myo*-inositol, 8 g l^{-1} agar (pH 5.7) and different plant growth regulators (PGRs) at various concentrations. The treatments consisted of combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration of 1 mg l^{-1} and either zeatin or N6-benzyladenine (BA) at concentrations 0.01, 0.05, or 0.1 mg l^{-1}. Of these media, two were adopted from the study of Correa et al. [12] in this experiment, namely 2,4-D (1 mg l^{-1}) in combination with BA (0.01 mg l^{-1}, or 0.1 mg l^{-1}). The media were denoted as A, B, C, D, E, F in order of appearance. Medium without PGRs was used as control. Cultures were maintained at 25/23°C under a 16/8 h light/dark regime with 36 μmol m^{-2} s^{-1} cool white fluorescent light. After 8 weeks of culture, proembryogenic calli were periodically subcultured onto the same medium every 20 d. A total of 20 explants were used per treatment in two replications.

The proembryogenic calli were transferred into Erlenmeyer flasks (100 ml) with 25 ml of embryo induction medium. Full strength MS medium without PGRs, containing 30 g l^{-1} sucrose, 1 mg l^{-1} thiamine and 100 mg l^{-1} *myo*-inositol and 8 g l^{-1} agar (pH 5.7) was used. The cultures were kept at 25/23°C under a 16/8 h light/dark regime. A total of ten calli were used per treatment in three replications.

In vitro regenerated plants were isolated and transferred individually to MS medium, and maintained together with control plants in a sustainable manner described above, with regular subcultures of 30–40 d. All *in vitro* experiments were arranged as a completely randomized design. Statistical analysis of data obtained from flow cytometric analysis, was performed by analysis of variance (ANOVA) and the significantly different means were identified by using the Tukey's HSD test ($p = 0.05$) [StatSoft STATISTICA 9.0].

2.3. Histological analysis of embryogenic structures

To comfirm regeneration of plants *via* somatic embryos, proembryogenic callus and somatic embryos at the cotyledonary stage were fixed by immersion in 50% FAA (formaldehyde:acetic acid:ethanol:water, 1:1:9:9) for 48–96 h. Dehydration was carried out according to standard procedures with tissues being passed through ethanol–butanol dehydration series, followed by embedding in paraffin wax at 58–60°C [24,25]. The paraffin blocks were cut into ribbons of thin sections (13 μm) with a rotary microtome (Thermo Shandon Finesse, UK). The microtome sections were stained with 0.1% alcian blue in 3% acetic acid and 0.1% nuclear fast red in 5% $Al_2(SO_4)_3$ [26] and photographed under a Nikon Eclipse 80i (Nikon, Japan) microscope equipped with a camera Nikon Digital Sight DS-5Mc (Nikon, Japan).

In total, 60 randomly selected regenerated plants were subjected to molecular and ploidy analysis. Evaluation was performed immediately after plant regeneration, and after the third subculture.

2.4. Flow cytometric analysis

Flow cytometry measurement was performed using a Partec II flow cytometer (Partec GmbH, Monster, Germany) equipped with an HBO mercury arc lamp. Sample preparation followed the two-step methodology according to Dolezel et al. [27]. Approximately 1 cm^2 of leaf tissue from both the sample and an appropriate amount of internal reference standard (*Bellis perennis* L., 2C = 3.38 pg; Schonswetteer et al. [28]) were chopped with razor blade in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a 42-μm nylon mesh and incubated for 10 min at room temperature. The staining solution consisted of 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented by AT-selective fluorescent dye 4′,6-diamino-2-phenylindol and 2-mercaptoethanol in final concentrations of 4 μg ml^{-1} and 2 μl ml^{-1}, respectively. After a 5 min incubation at room temperature, the samples were analysed with a flow cytometer. The stained nuclei were analysed at a concentration of 5000 per sample. Obtained histograms were evaluated using the FloMax software (ver. 2.4d; Partec GmbH, Münster, Germany). For each analysed sample, DNA-ratios were counted by dividing the mean of the dominant (G0/G1) peak of the yacon sample by the mean of the G0/G1 peak of the internal standard. Coefficient of variation of the G0/G1 peaks was also recorded.

2.5. DNA extraction and ISSR analysis

Total DNA was extracted from leaf material obtained from 60 regenerated plantlets and initial plant material was used as control. From the fresh leaves were taken the samples of about 100 mg weight. For the extraction of genomic DNA the Invisorb® Spin Plant Mini Kit (Stratec Molecular, Germany) was used. Twenty ISSR primers (The University of British Columbia Biotechnology Laboratory, Canada) were tested. Polymerase chain reaction (PCR) amplifications were performed in a 20 μl reaction volume containing 1 μl of template DNA at concentration 50 ng μl^{-1}, 0.5 μl of primer at concentration 0.5 μM, 10 μl PPP Master Mix (Top-Bio, Czech Republic), 0.2 μl BSA (Fermentas, Germany), 8.3 μl PCR H_2O (Top-Bio, Czech Republic). Amplifications were performed in a T100™ Cycler (Bio-Rad, USA). The PCR was carried out with modifications of the annealing temperature to optimize the

Table 1
Primers used in ISSR polymorphism analysis, number and size of amplified fragments.

Primers code (UBC)	Sequence 5′–3′	Annealing temperature (°C)	Total number of bands amplified	Number of scorable bands per primer	No. and frequency of polymorphic bands per primer	Polymorphic regenerants	Range of amplification (pb)
UBC807	$(AG)_8T$	46.5	420	7	0		350–1000
UBC809	$(AG)_8G$	48.0	420	7	0		450–1250
UBC810	$(GA)_8T$	50.0	540	9	0		300–1350
UBC823	$(TC)_8C$	47.5	232	4	2 (50%)	A6, E1, E9, F5, F9	700–1300
UBC824	$(TC)_8G$	48.0	600	10	0		300–1100
UBC828	$(TG)_8A$	50.0	420	7	0		550–2500
UBC829	$(TG)_8C$	52.5	480	8	0		500–1300
UBC834	$(AG)_8YT$	52.0	239	4	1 (25%)	F5	300–1200
UBC835	$(AG)_8YC$	50.0	420	7	0		350–2500
UBC836	$(AG)_8YA$	48.0	423	9	1 (11%)	B8, E1, F5	500–2000
UBC840	$(GA)_8YT$	46.5	478	8	1 (11%)	E9, F5	180–1000
UBC841	$(GA)_8YC$	52.0	423	9	2 (22%)	A6, F5	200–1300
UBC844	$(CT)_8RC$	47.5	297	5	1 (20%)	A6, E9, F5	600–1250
UBC845	$(CT)_8RG$	47.5	179	3	1 (33%)	F5	800–1300
UBC847	$(CA)_8RC$	52.5	360	6	0		550–2000
UBC851	$(GT)_8CT\ G$	52.5	540	9	0		650–1900
UBC854	$(TC)_8RG$	50.0	180	3	0		500–1300
UBC855	$(AC)_8YT$	53.0	360	10	0		700–1350
UBC856	$(AC)_8YA$	54.0	597	10	1 (10%)	A6, E9, F5	500–1300
UBC873	$(GACA)_4$	46.5	240	4	0		1000–1300
Total			7848	139	10 (7.19%)		

reaction for individual primers. The cycling conditions were as follows: initial denaturation step for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C (denaturing), 1 min at specific annealing temperature (Table 1), 2 min at 72°C (extension), and 1 cycle for 10 min final extension step at 72°C. Electrophoretic separation was performed with 5.5 μl of amplified products on 2% agarose gel in 1× TBE buffer. Gels were run for about 3–3.5 h at 55 V. DNA amplification products were stained with SYBR® Safe DNA Gel Stain (Invitrogen, USA).

The PCR reaction for each ISSR primer was performed in two repetitions, only clear and completely reproducible bands were included in data evaluation. The bands were scored as presence (1) and absence (0) for each regenerant and control plant and were transformed into a binary character matrix. Genetic dissimilarity was calculated with Jaccard's distances using Darwin 5.0 software [29].

3. Results and discussion

In our experiment, callus from petiole segments started to appear by the 3rd week on all initiation media tested. These calli were yellowish-green, friable and fast growing. Histological examination confirmed numerous meristematic tissues consisting of small and dense cells in the callus, suggesting proembryogenic structure formation (Fig. 1a). The induction frequency of proembryogenic callus in the treatments varied from 70 to 100%. The maturation of somatic embryos and development into plantlets was achieved after transfer of proembryogenic calli to induction media without auxin as was reported in the previous study by Correa et al. [12]. Histological analysis confirmed the bipolar character of developed structures and showed no vascular connection with the callus. Successful regeneration of plants *via* somatic embryogenesis (Fig. 1b, c) was achieved from each of the tested initiation media and *in vitro* plantlets did not show any evident morphological abnormalities. Ten randomly selected plants from the six treatments were subjected to ISSR and flow cytometric analysis to reveal potential somaclonal variation.

3.1. ISSR and flow cytometric analysis

All 20 tested ISSR primers provided clear and scorable bands with satisfactory intensity. A total of 7848 bands were generated from the control plant and 60 *in vitro* regenerants. The size of the amplification fragments ranged from 180 to 2500 bp. The number of bands for each primer varied from 3 to 10, and an average of 6.95 bands was obtained per ISSR primer. Eight tested primers generated polymorphic bands, most of which provided one polymorphic band from their DNA profiles. Only 'UBC823' and 'UBC841' generated two polymorphic bands. Fig. 2 shows polymorphic amplification patterns obtained with ISSR primer 'UBC834'. The total polymorphism scored is given in Table 1. In four initiation media tested (*i.e.* A, B, E and F) at least one variable regenerant in a different primer fragment profile was revealed. Regenerant F5 was detected as the most variable, showing polymorphism in primers `UBC823', `UBC834', `UBC836', `UBC840', `UBC841', `UBC844', `UBC845' and `UBC856'. In most primers, polymorphic bands were absent in the DNA pattern of variable regenerants when compared to that one of the control plant, with the exception of primers 'UBC836' and 'UBC841' where extra bands were scored.

The ISSR data was used to calculate the Jaccard's distances. It revealed rather lower genetic variability in tested samples, *i.e.* 0.104, 0.020, 0.040, 0.106, 0.163 and 0.040 for the regenerants A6, B8, E1, E9,

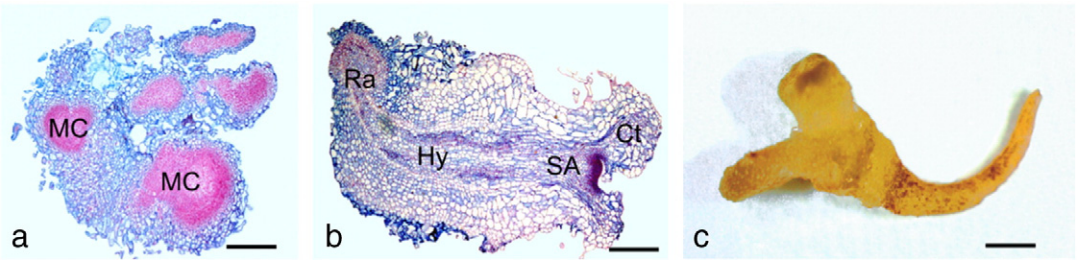

Fig. 1. Somatic embryogenesis in yacon. (a) Proembryonic callus with meristematic centres (*bar*, 0,5 mm). (b) Early cotyledonary embryo (*bar*, 0.8 mm). (c) Germinated well developed embryo (*bar*, 1.5 mm). (Ct: Cotyledon; Hy: Hypocotyl; ME: Meristematic Centres; Ra: Radicle; SA: Shoot Apex).

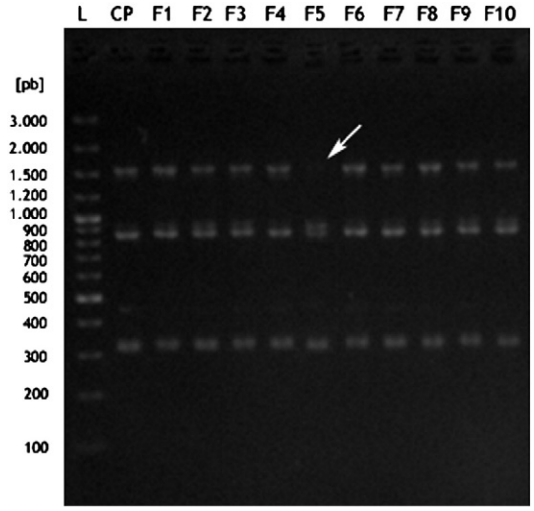

Fig. 2. ISSR profile of control plant and *in vitro* regenerants (F1–F10) of *S. sonchifolius* using primer 'UBC834' (L: ladder; CP: control plant).

F5 and F9, respectively. The other regenerants had a distance value of 0 compared to the control plant, indicating no genetic variation.

ISSR analysis was found to be a reliable method, enabling rapid evaluation of somaclonal variability by fast scanning of the whole genome as reported by Rathore et al. [30]. In our case, the method revealed genetic variability in 6 among 60 plants under study. Eight primers were polymorphic and generated 10 polymorphic bands with 7.19% mean polymorphism. Similarly, many recent studies have used ISSR analysis to determine somaclonal variation of *in vitro* regenerants, *e.g.* in *Nothapodytes foetida* 7.53% polymorphism [31], in *Aloe vera* 15.1% polymorphism [30], and in *Cymbopogon pendulus* 9.4% polymorphism [32] was detected. Since ISSR analysis is not effective in detection of epigenetic changes, Linacero et al. [33] applied a modified method of ISSR using methylation sensitive restriction enzymes to digest DNA, followed by PCR amplification, to detect both epigenetic and genetic changes in somatic-embryo derived plants of *Secale cereale*. In yacon, however, detailed research on epigenetic changes still need to be carried out.

PCR-based markers can be used for genetic discrimination purposes to locate and isolate mutations linked to these markers [19], however, some important variations like genomic mutations may not be detected [34]. For this reason, ploidy level detection is a useful complementary approach [19].

Linear histograms of relative nuclear DNA content showed in all cases two peaks, the first corresponding to somatic nuclei arrested in the G0/G1 phase of the cell cycle, and belonging to the internal standard *Bellis perennis*, and the second representing nuclei of the yacon sample in the G0/G1 phase (Fig. 3a, b). The DNA-ratios of regenerated plantlets, obtained from different initiation media, varied from 1.708 to 1.722, and they were not significantly different to that of the control plant (1.709), suggesting that the plants obtained *via* somatic embryogenesis had maintained stable octoploid level. Coefficients of variation did not exceed 2.48 in any of the measurements. Absence of alterations in ploidy level indicates that there is no correlation between molecular and genomic changes.

Somaclonal variation in terms of genome changes is usually associated with aneuploidy or polyploidy [35]. In many diploid and tetraploid species, increase of ploidy level in regenerants was reported, especially when plants were recovered from long-term callus or suspension cultures [20,36]. Octoploid yacon, however, seems to be stable and not susceptible to ploidy level alteration, as demonstrated also in a previous study where extremely low frequency of polyploids was obtained after *in vitro* induction of polyploidization by means of colchicin and oryzalin [37]. Moreover, histograms in the present study showed only two peaks, indicating no polysomaty. Thus, pre-existence of ploidy variability in initial explants might be excluded.

To our best knowledge, no study on somaclonal variation in plants of yacon regenerated *via* somatic embryogenesis has been made before. ISSR markers proved to be a powerful technique to reveal somaclonal variation. Based on our results, it can be concluded that somatic embryogenesis probably may not be favourable for large-scale propagation and conservation purposes in yacon, as the risk of genetic instability cannot be excluded. Nevertheless, it seems to be an effective technique to widen genetic variability and it can be used in yacon improvement, especially when low sexual reproductive capacity hinders classical ways of breeding. Besides, somatic embryos may serve as a valuable source for studies of biochemical, physiological and morphological processes during embryo developmental stages, as the lack of zygotic embryos in yacon does not allow us to carry out this type of research.

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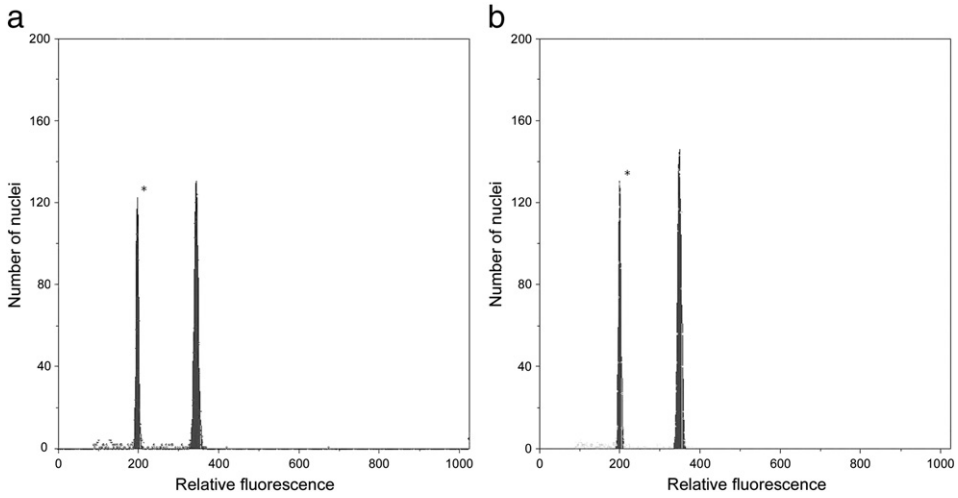

Fig. 3. Representative flow cytometric histograms documenting the relative DNA content of *in vitro* plants. (a) Control plant; (b) Randomly selected *in vitro* regenerant. The peak indicated as "*" corresponds to the internal reference standard (*Bellis perennis*).

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