



# Genetic stability of micropropagated plants of *Crambe abyssinica* Hochst using ISSR markers

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**ABSTRACT.** *Crambe* (*Crambe abyssinica*) is a non-edible annual herb, which was first cultivated to extract oil for industry, and now has great potential for biodiesel production. The objective of this investigation was to evaluate the genetic stability of micropropagated plants of the *C. abyssinica* Hochst cultivar 'FMS brilhante' using polymerase chain reaction techniques based on inter-simple sequence repeat (ISSR) molecular markers. The aim was to develop a protocol for the *in vitro* regeneration of these plants with low genetic variation as compared to the donor plant. For micropropagation, shoot tips from *in vitro* germinated seedlings were used as explants and were initially cultivated for 90 days on MS medium with 5.0  $\mu$ M 6-benzylaminopurine (BAP), which at 90 days, led to the highest

number of shoots per explant (NSE) (12.20 shoots) being detected. After 120 days, the interaction between BAP concentration and naphthalene acetic acid (NAA) was tested, and the highest NSE was observed following exposure to 0.0/0.5  $\mu\text{M}$  BAP/NAA (11.40 shoots) and 1.0/0.0  $\mu\text{M}$  BAP/NAA (11.00 shoots). The highest proportion of rooting phase were observed following exposure to 0.5  $\mu\text{M}$  NAA (30%). The 13 ISSR primers used to analyze genetic stability produced 91 amplification products, of which only eight bands were polymorphic and 83 were monomorphic for all 10 regenerated crambe plants, compared to the donor plant explant. These results indicate that crambe shoot tips are a highly reliable explant that can be used to micropropagate genetically true-to-type plants or to maintain genetic stability, as verified using ISSR markers.

**Key words:** *In vitro*; Regeneration; Somaclonal variation; Shoot tips; Molecular markers; Micropropagation

## INTRODUCTION

*Crambe abyssinica* (Brassicaceae;  $2n = 6x = 90$ ), herein referred to as “crambe”, is a non-edible annual herb native to the Mediterranean region, which was initially grown for industrial oil extraction and has high potential for biodiesel production. Oil extracted from its seeds has a high erucic acid content (50-60%), which is used to produce lubricants, plastics, corrosion inhibitors, synthetic rubbers, and cosmetic products (Lara-Fioze et al., 2013).

Additionally, the agronomic characteristics of crambe, such as its short life cycle of approximately 90 days, high biomass, mean seed yield of 1800-2400 kg/ha, adaptation to different climatic conditions, resistance to insects, and tolerance to abiotic stresses such as salinity, low temperature, heat, drought, and exposure to heavy metals, make it an important species within the agricultural industry (Paulose et al., 2010; Chhikara et al., 2012).

However, for the crambe crop to be employed effectively, biotechnological interventions are required to supply quality plant material. In this context, *in vitro* cell and tissue culturing techniques are important both for mass propagation (Li et al., 2010; Li et al., 2011; Palmer and Keller, 2011; Chhikara et al., 2012) and as a tool for genetic improvement (Wang et al., 2004; Wang et al., 2006).

Effective plant regeneration using *in vitro* tissue culturing techniques is a prerequisite for the application of biotechnological tools to improve the crop (Singh et al., 2012). Micropropagation studies have documented crambe regeneration *in vitro* via direct (Li et al., 2010; Li et al., 2011) and indirect organogenesis (Chhikara et al., 2012), using hypocotyls as explants for both, and via indirect somatic embryogenesis (Palmer and Keller, 2011) with cotyledons, hypocotyls, and roots as explants. However, no published data describe crambe regeneration using apical segments via multiplication by axillary bud proliferation.

Maintaining genetic stability of plants regenerated *in vitro* relative to the mother plant (explant donor) is important to enable subtle phenotypic, cytological, biochemical, and/or molecular variations to be detected (Borchetia et al., 2009), thus enabling strategic planning for the propagation of species with desired quality and uniformity before the plants are released for commercial purposes (Bhatia et al., 2009).

In the case of micropropagation methods involving pre-existing meristems, such as axillary buds and apical meristems, studies have reported that different plant species have maintained

genetic stability after the *in vitro* stages. However, studies have also reported the presence of genetic, epigenetic, and phenotypic variations, known as somaclonal variations, which occur during *in vitro* regeneration processes (organogenesis and embryogenesis) (Sharma et al., 2011; Pathak and Dhawan, 2012; Singh et al., 2012).

In this sense, molecular markers, especially those based on DNA, are powerful and valuable tools that can analyze genetic stability in plants propagated *in vitro* (Gheorghe et al., 2009). Among these markers, the use of inter-simple sequence repeats (ISSR) to evaluate genetic stability is well documented (Leroy et al., 2001; Bhatia et al., 2009) and, according to Leroy et al. (2001), these are considered adequate to detect variations derived from tissue cultures among plants of the family Brassicaceae, such as cauliflower (*Brassica oleracea*), rapeseed (*Brassica napus*), radish (*Raphanus sativus*), and crambe (*C. abyssinica*).

To our knowledge, none of these studies investigating *in vitro* propagation of crambe has reported the genetic stability of regenerated plants. Therefore, in the present study, we aimed to test the genetic stability of plants micropropagated from *C. abyssinica* Hochst cultivar FMS Brilhante using ISSR molecular markers.

## MATERIAL AND METHODS

### Plant materials

The experiments were performed at the Laboratory of Plant Tissue Culture and at the Laboratory of Plant Biochemistry and Molecular Biology of the Centre of Agricultural Sciences at the Federal University of Espírito Santo (Universidade Federal do Espírito Santo - UFES), Brazil. *C. abyssinica* Hochst seeds (FMS Brilhante cultivar) from a 2012 harvest were obtained from the MS Foundation (Fundação MS), located in Maracaju, Mato Grosso do Sul, Brazil, and were used to establish an *in vitro* culture.

### Explant source

Apical segments were excised from crambe seedlings germinated from seeds *in vitro*. The seeds were first washed under running water with neutral detergent and then sterilized by immersion in a solution containing penicillin (10 mg/L) and rifampicin (10 mg/L) for 30 min under aseptic conditions in a laminar flow chamber. The segments were then washed in 70% ethanol for 1 min, followed by submersion in 50% commercial sodium hypochlorite (active chlorine: 2-2.5%) for 30 min, and washed in autoclaved distilled water three times.

### Culture medium and culturing conditions

Culture medium consisting of ½MS (Murashige and Skoog, 1962) supplemented with 15 g/L sucrose and 7 g/L agar, was used to germinate the crambe seeds *in vitro*. After 30 days, adequate apical segments were obtained and used in the micropropagation process.

To induce shoots, the explants were cultured for 90 days in MS medium with 5 µM 6-benzylaminopurine (BAP), supplemented with 30 g/L sucrose, 0.5 mg/L silver nitrate (AgNO<sub>3</sub>) (Li et al., 2010), and 7 g/L agar. The cultures were subcultured and tests were performed at 30, 60, and 90 days after inoculation. Starting from the third subculture (90 days), the regenerated shoots were treated to reduce the concentration of cytokinin before auxin was added to the medium. For

this, the effect of the interaction between different concentrations of BAP (0.0, 1.0, 3.0, and 5.0  $\mu\text{M}$ ) and 1-naphthaleneacetic acid (ANA) (0.0 and 0.5  $\mu\text{M}$ ) was tested using MS medium, with 30 g/L sucrose, 0.5 mg/L  $\text{AgNO}_3$ , and 7 g/L agar. Shoots were evaluated and subcultured after 30 days (total time 120 days).

The rooting stage was only applied to the control (0.0  $\mu\text{M}$ ) and to the three treatments that exhibited shoots with a better morphological appearance, which were obtained from the interaction between BAP and ANA (3.0/0.0, 5.0/0.0, and 1.0/0.5  $\mu\text{M}$  BAP/ANA). For this stage,  $\frac{1}{2}$ MS medium was first used with 0.5  $\mu\text{M}$  ANA. Next, the shoots were transferred to  $\frac{1}{2}$ MS medium with 1.0  $\mu\text{M}$  ANA or 0.1  $\mu\text{M}$  BAP, both supplemented with 10 g/L sucrose, 100 mg/L penicillin (Li et al., 2010; Chhikara et al., 2012), 0.5 mg/L  $\text{AgNO}_3$ , and 7.5 g/L agar. The test was performed after 30 days in the ANA or BAP rooting medium as described, at 150 and 180 days, respectively.

The pH of the media was adjusted to 5.8, and then transferred into 10-mL test tubes and autoclaved at 1.1 atm (standard atmosphere), 121°C, for 20 min. All cultures were incubated in a growth room at  $25 \pm 1^\circ\text{C}$ , under a 16/8-h photoperiod (light/dark), and fluorescent lights supplying  $25.2 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  photosynthetic photon.

### Morphological (phytotechnical) evaluations

Morphological evaluations were performed at different micropropagation stages at 30, 60, 90, 120, 150, and 180 days to determine the number of shoots per explant (NSE), mean shoot length (MSL) by digital caliper, rooting percentage (rooting%), and survival percentage (survival%).

### Genetic stability assessment

The genetic stabilities of the explant donor plant and 10 plants regenerated *in vitro* were analyzed using ISSR molecular markers. After excising the apical segment at the onset of the micropropagation process, the explant donor plant was numerically labeled and frozen in aluminum foil in an ultrafreezer at  $-30^\circ\text{C}$ ; all shoots generated from this explant were labeled with the number corresponding to their origin. The 10 regenerated plants were removed from  $\frac{1}{2}$ MS medium at the rooting stage (180 days).

Genomic DNA was extracted from the entire plant according to the method described by Doyle and Doyle (1990) with modifications as proposed by Abdelnoor et al. (1995). The DNA concentration was determined using a Nanodrop (Thermo scientific), and the DNA quality was verified by electrophoresis on a 0.8% agarose gel. After extraction, the DNA was stored at  $-30^\circ\text{C}$  until use.

Twenty ISSR primers from the University of British Columbia (UBC, Vancouver, Canada) were tested, and 13 were selected for use in the genetic stability assay due to their reproducibility, band clarity, and exhibition of polymorphisms. The ISSR amplifications were performed in an Applied Biosystems® thermocycler, in a final volume of 25  $\mu\text{L}$  containing 30 ng genomic DNA, 0.2  $\mu\text{mol/L}$  each primer, 0.25 mM each dNTP, 2.5 mM  $\text{MgCl}_2$ , 0.25 mM Tris-KCl (pH: 8.3), and 1 U Taq polymerase. The amplification conditions for the primers UBC 810, 834, 841, 849, 855, 857, 859, 864, 879, 889, and 890 were as follows: initial denaturation at  $94^\circ\text{C}$  for 15 min; followed by 35 cycles composed of three steps, a) denaturation at  $94^\circ\text{C}$  for 30 s, b) primer annealing at  $52^\circ\text{C}$  for 30 s, and c) extension at  $72^\circ\text{C}$  for 1 min; with a final extension step at  $72^\circ\text{C}$  for 7 min. The following protocol was used for the primers UBC 827 and 845:  $94^\circ\text{C}$  (15 min); followed by 35 cycles at  $94^\circ\text{C}$  for 1 min,  $52^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min; and then, a final extension stage at  $72^\circ\text{C}$  for 7 min.

The amplified DNA fragments were separated by electrophoresis on a 2.5% agarose gel containing 0.02  $\mu\text{L/mL}$  ethidium bromide in 1X TBE buffer at 110 volts for approximately 3-4 h. A

DNA molecular weight marker (100-bp ladder, Fermentas®) was used to estimate the molecular mass of the fragments obtained. After the run, gels were photographed under ultraviolet light using the L PIX HE photo documentation system (Loccus Biotecnologia®). The gels were then analyzed for the presence/absence of bands between donor and regenerated plants for each primer.

## Experimental design and statistical analysis

The experiments were conducted using a completely randomized design (CRD). The shoot inductions with 5 µM BAP performed at 30, 60, and 90 days consisted of 50 replicates per time point, consisting of one explant each. The effects of the interaction between the concentrations of BAP and ANA were evaluated in a 4 x 2 factorial scheme (0.0, 1.0, 3.0, and 5.0 µM BAP x 0.0 and 0.5 µM ANA) with five replicates, consisting of five explants each. The same number of replicates and explants were used in the rooting experiment. The rooting% data were transformed into  $[(x/100) + 1]$ .

All data obtained were subjected to analysis of variance (ANOVA) after the normality and homogeneity were tested. If the treatment was found to be significant, Tukey's test was used, adopting 1 and 5% probability levels using the software Assistat (Silva and Azevedo, 2009).

## RESULTS AND DISCUSSION

Apical segments (Figure 1B) from the seedlings germinated *in vitro* (Figure 1A) and cultured in MS medium with 5.0 µM of BAP began inducing shoots within ~2 weeks of culturing. The morphological appearance of the shoots regenerated at 30 days (Figure 1C) demonstrated that BAP was effective at inducing new shoots and breaking the apical dominance. At 60 and 90 days, there was a shrub-like appearance (Figure 1D and E, respectively).

The mean values of the characteristics evaluated in the explants under the influence of this growth regulator (BAP) at 30, 60, and 90 days are shown in Table 1. The NSE was significantly affected by the culture duration, and at 90 days the value was higher (12.20 shoots) than that at 30 and 60 days (8.08 and 8.46 shoots, respectively). The dose of cytokinin in the culture medium was sufficient to induce shoots in crambe, which is critical for effective organogenesis. The effect of BAP on the induction of multiple shoots has been previously reported in species of the family Brassicaceae (Shukla and Sawhney, 1991; Memon et al., 2009), but no studies have described the effect of this regulator over time *in vitro* cultures.

**Table 1.** Mean number of shoots per explant (NSE), mean shoot length (MSL), and survival percentage (survival%) in apical segments of *Crambe abyssinica* at a BAP concentration of 5.0 µM after 30, 60, and 90 days of culturing *in vitro*.

Time (days)	NSE	MSL (mm)	survival%
30	8.08 <sup>b</sup>	27.68 <sup>a</sup>	92.00 <sup>a</sup>
60	8.46 <sup>b</sup>	28.00 <sup>a</sup>	90.00 <sup>b</sup>
90	12.20 <sup>a</sup>	28.86 <sup>a</sup>	91.00 <sup>a</sup>
Overall mean	9.58	28.18	91.00
CV (%)	33.30	22.14	32.34

Means followed by the same letter in the column do not differ by Tukey's test at 5% CV(%): percent coefficient of variation.

The MSL did not differ significantly at 30, 60, and 90 days and reached a mean of 28.18 mm after 90 days of culturing (Table 1). This shows that although there were subcultures, the

excised shoots maintained a similar growth rate. During treatment with 5  $\mu\text{M}$  BAP, no roots formed, indicating the need to add an auxin to elicit such a response. The survival percentage was, on average, 91% during this 90-day period; losses occurred due to fungal contamination.

Next, interactions between different concentrations of BAP and ANA in the explants were tested, and the morphological appearance was assessed at 120 days (Figure 1F). The results clearly indicate that the regenerative ability of the shoots continued with values close to those found at the earlier evaluation times.

Among the treatments employed to investigate the interaction between BAP and ANA at 120 days, the highest NSE occurred with 0.0/0.5  $\mu\text{M}$  BAP/ANA (11.40 shoots) and 1.0/0.0  $\mu\text{M}$  BAP/ANA (11.00 shoots) (Table 2). However, when different BAP concentrations were tested with 0.0  $\mu\text{M}$  ANA, the multiplication of shoots was higher at 1.0  $\mu\text{M}$  BAP (11.00 shoots), without significantly differing from that observed at 0.0 and 3.0  $\mu\text{M}$  BAP. When different concentrations of BAP were used with 0.5  $\mu\text{M}$  ANA, the test with 0.0  $\mu\text{M}$  BAP led to the highest value (11.40 shoots), with no difference observed between the effect at 1.0  $\mu\text{M}$  BAP. The presence of 0.5  $\mu\text{M}$  ANA resulted in a significantly different value for NSE compared to that observed in its absence when employed without BAP. Thus, the lowest BAP concentrations (0.0 and 1.0  $\mu\text{M}$ ) in the absence or presence of 0.5  $\mu\text{M}$  of ANA had a more positive affect on the induction of shoots at 120 days. Of note, this was the time point when signs of a decline in NSE were observed during treatment with 5.0  $\mu\text{M}$  BAP after 90 days.

**Table 2.** Mean number of shoots per explant (NSE) of *Crambe abyssinica* seedlings under different interacting concentrations of BAP and ANA after 120 days of culturing *in vitro*.

BAP ( $\mu\text{M}$ )	ANA ( $\mu\text{M}$ )	
	0	0.5
0.0	8.80 <sup>abB</sup>	11.40 <sup>aA</sup>
1.0	11.00 <sup>aA</sup>	8.80 <sup>abA</sup>
3.0	8.60 <sup>abA</sup>	6.40 <sup>aA</sup>
5.0	7.00 <sup>aA</sup>	7.80 <sup>aA</sup>

Means followed by the same lowercase letter in the columns and uppercase letter in the rows do not differ by Tukey's test at 5%.

A positive effect of BAP on the regeneration of a wide range of explants has been previously reported by Li et al. (2011), when 10  $\mu\text{M}$  thidiazuron (TDZ) and 2.5  $\mu\text{M}$  ANA were used and 95% shoot regeneration was reported in hypocotyls of the *C. abyssinica* cultivar Galactica. These results demonstrate that the balance between cytokinins and auxins is important, and that it affects the morphogenetic response *in vitro*. According to Shukla and Sawhney (1991), the presence of both BAP and ANA stimulates shoot production in several types of *Brassica* explants, but the response depends on the concentration used. Vicente et al. (2009) also reported that high BAP concentrations reduced the NSE, most likely due to the phytotoxic effect of the cytokinin, which is consistent with the observed reduction in NSE at 120 days in the presence of 5  $\mu\text{M}$  BAP.

For rooting, in addition to the control, the shoots treated with different concentrations of BAP and ANA that had better morphological appearances and intermediate NSE values and no calluses at their bases were selected. Thus, shoots treated with 0.0/0.0, 3.0/0.0, 5.0/0.0, and 1.0/0.5  $\mu\text{M}$  BAP/ANA were first treated with 0.5  $\mu\text{M}$  ANA for 30 days (150 days) and then with 1.0  $\mu\text{M}$  ANA + 0.1  $\mu\text{M}$  BAP for a further 30 days (180 days). Low rooting rates were observed in these media, and there were no significant differences among the rates (Table 3). The highest rooting rates were observed in the control, 30% was observed with 0.5  $\mu\text{M}$  ANA, and 20% was observed with 1.0  $\mu\text{M}$  ANA + 0.1  $\mu\text{M}$  BAP (Figure 1G and H).

**Table 3.** Effect of 0.5  $\mu\text{M}$  ANA (150 days) and 1.0  $\mu\text{M}$  ANA + 0.1  $\mu\text{M}$  BAP (180 days) on the number of shoots per explant (NSE), rooting percentage (rooting%), and survival percentage (survival%) in *Crambe abyssinica* seedlings from *in vitro* culturing.

Treatments	0.5 $\mu\text{M}$ ANA (150 days)			1.0 $\mu\text{M}$ ANA + 0.1 $\mu\text{M}$ BAP (180 days)		
	NSE	rooting%	survival%	NSE	rooting%	survival%
0.0 $\mu\text{M}$	6.35 <sup>b</sup>	30.00 <sup>a</sup>	100.00	7.96 <sup>c</sup>	20.00 <sup>a</sup>	95.00 <sup>a</sup>
3.0 $\mu\text{M}$ BAP	16.40 <sup>a</sup>	25.00 <sup>a</sup>	100.00	14.45 <sup>ab</sup>	10.00 <sup>a</sup>	100.00 <sup>a</sup>
5.0 $\mu\text{M}$ BAP	16.20 <sup>a</sup>	10.00 <sup>a</sup>	100.00	13.41 <sup>b</sup>	10.00 <sup>a</sup>	95.00 <sup>a</sup>
1.0 $\mu\text{M}$ BAP + 0.5 $\mu\text{M}$ ANA	15.65 <sup>a</sup>	5.00 <sup>a</sup>	100.00	18.70 <sup>a</sup>	0.00 <sup>a</sup>	100.00 <sup>a</sup>
Overall mean	13.65	17.50	100.00	13.63	10.00	97.50
CV (%)	16.57	15.78	-	19.36	12.96	8.11

Means followed by the same lowercase letter in the columns do not differ by Tukey's test at 5%.

Memon et al. (2009) reported 90% rooting of *Brassica campestris* shoots in MS medium supplemented with 1  $\mu\text{M}$  ANA, whereas only 54.4% rooting was observed when the medium was supplemented with 2.5  $\mu\text{M}$  ANA. The results of Li et al. (2010) using adventitious shoots generated from the hypocotyls of the *C. abyssinica* cultivar Galactica were not similar to those reported in the present study. These authors reported that in the presence of 0.54 and 0.27  $\mu\text{M}$  ANA, rooting values were 59 and 67%, respectively, whereas 0.49  $\mu\text{M}$  AIB and 0.25  $\mu\text{M}$  AIB led to low rooting frequencies (24 and 18%, respectively). Thus, most likely, the long culturing duration, excess BAP, and short shoot length made rooting inefficient in the present study.

These results suggest that it is necessary to add a rooting cofactor to promote rhizogenesis in explants. The addition of phenolic compounds to the culture medium, such as phloroglucinol and/or phloretic acid (Mendes et al., 2007), activated charcoal (Lonardo et al., 2013), or dark charcoal (Paiva Neto et al., 1998), or antioxidants (Souza and Pereira, 2007), together with auxins, can increase the rooting percentage in shoots of crambe cultured *in vitro* with high BAP concentrations over a prolonged duration.

Regarding the NSE at 150 and 180 days, the highest values were observed in seedlings treated with a growth regulator, for which the highest values were found following treatment with 3.0/0.0  $\mu\text{M}$  BAP/ANA at 150 days (16.40 shoots) and with 1.0/0.5  $\mu\text{M}$  BAP/ANA at 180 days (18.70). These results demonstrate that despite the removal of BAP and the addition of ANA alone to induce rooting, higher NSE values were observed compared to those detected at other time points. Therefore, there is a prolonged residual effect of BAP that directly affects the rooting stage. According to Qin et al. (2007), the use of auxins and cytokinins in culture medium is essential for efficient shoot induction and formation. Those authors used BAP alone and in combination with ANA, noting that higher shoot formation was obtained in media containing a combination of both. The same finding was demonstrated in the present study with crambe, for which the largest shoot and shoot length values were recorded after ANA was added to the culture medium.

The genetic stability of plants exposed to 3.0/0.0  $\mu\text{M}$  BAP/ANA (Figure 2, individuals 1-8) and 1.0/0.5  $\mu\text{M}$  BAP/ANA (Figure 2, individuals 9-10) was assessed, because they exhibited a better appearance during the rooting stage in  $\frac{1}{2}$ MS medium (180 days). All 10 of the regenerated plants assessed originated from the same explant donor plant (Figure 2, individual D).

Of the 20 ISSR markers used for the initial analysis, only 13 primers produced visible, reproducible, countable bands. These 13 primers generated 91 amplification products ranging from 200 to 1000 bp. The number of bands for each primer ranged from 1 to 11, with a mean of seven bands per ISSR primer. Of the total bands amplified, only eight were polymorphic; thus, 83 were monomorphic for the 10 regenerated crambe plants compared to the explant donor plant (Table 4).

The band profile obtained between the progeny and the regenerated plants demonstrated high uniformity for both treatments tested herein for crambe micropropagation, which suggests there is a high degree of genetic fidelity. These results confirm that crambe plants micropropagated using apical segments as explants maintain genetic stability, even after a prolonged period of 180 days, under the effects of growth regulators, such as BAP and ANA, and under stressful conditions during *in vitro* culturing.

**Table 4.** List of different UBC primers used to determine the genetic stability of *Crambe abyssinica* plants regenerated *in vitro*.

UBC Primer	Nucleotide sequence 5'-3'	No. of monomorphic bands	No. of polymorphic bands	Total amplified bands
UBC 810	GAGAGAGAGAGAGAT	3	1	4
UBC 827	ACACACACACACACG	8	3	11
UBC 834	AGAGAGAGAGAGAGYT	11	0	11
UBC 841	GAGAGAGAGAGAGAYC	4	0	4
UBC 845	CTCTCTCTCTCTCTRG	2	0	2
UBC 849	GTGTGTGTGTGTGTYA	8	0	8
UBC 855	ACACACACACACACACYT	8	0	8
UBC 857	ACACACACACACACACYG	5	0	5
UBC 859	TGTGTGTGTGTGTGRC	7	0	7
UBC 864	ATGATGATGATGATGATG	9	2	11
UBC 879	CTTCACTTCACTTCA	1	0	1
UBC 889	DBDACACACACACAC	9	0	9
UBC 890	VHVGTTGTGTGTGTGT	8	2	10
Total number of bands produced		83	8	91

The use of apical or nodal segments for micropropagation is an important method that has been used successfully in many plant cultures (Sharma et al., 2011) due to its simplicity, low risk of genetic instability, and high propagation rate (Singh et al., 2012). Although transfers and long duration *in vitro* are known to induce somaclonal variations, there are limited molecular data on the effect of the number of subcultures on the stability of micropropagated plant material (Borse et al., 2011).

The pattern of DNA amplification observed in the cultured crambe plants is shown in the gel profiles containing the primers UBC 810, 827, 834, 841, 849, 855, 857, 859, 864, and 890 (Figure 2A-J).

The primers UBC 834 (Figure 2C), 841 (Figure 2D), 845, 849 (Figure 2E), 855 (Figure 2F), 857 (Figure 2G), 859 (Figure 2H), 879, and 889 were entirely monomorphic for the donor plant and its regenerated plants. The primers UBC 810 (Figure 2A), 827 (Figure 2B), 864 (Figure 2I), and 890 (Figure 2J) exhibited polymorphisms with up to three bands being observed (Table 4). The primers that exhibited higher levels of amplification product were UBC 827, 834, and 864, among which UBC 834 was entirely monomorphic.

Culturing *in vitro* may induce the loss of cellular control, resulting in somaclonal variations (Pathak and Dhawan, 2012). The reasons accounting for somaclonal variations remain unclear, although factors such as culture duration, number of subcultures or transfers (Borse et al., 2011), phytohormones (Biswas et al., 2009), explant type (Chuang et al., 2009), passage through the indirect callus phase (mass of undifferentiated cells with regeneration potential) (Peredo et al., 2006), genotype (Khan et al., 2009), culture medium composition (Lutts et al., 1998), and ploidy and mosaicism levels (Nakano et al., 2006) are considered capable of inducing this variability *in vitro*.

The possible mechanisms involved include chromosomal and point mutations, somatic recombination, sister chromatid exchange, somatic genetic rearrangement, transposable element activity, tandem repeats, sequence instability, and epigenetic processes, such as DNA methylation (Pathak and Dhawan, 2012).

Crambe tissue culturing has been used to propagate (Li et al., 2011; Palmer and Keller, 2011), genetically improve (Li et al., 2010; Chhikara et al., 2012; Li et al., 2013), and to identify and use some of its genes to improve other species (Wang et al., 2004; Wang et al., 2006). The use of molecular markers in crambe reported in the literature is limited to the study of genetic diversity (Lara-Fioze et al., 2013), identification and characterization of genetic transformants (Wang et al., 2004; Wang et al., 2006; Li et al., 2010; Xu et al., 2010; Chhikara et al., 2012; Li et al., 2013), and identification of genes of interest (Paulose et al., 2010; Cheng et al., 2013). Therefore, the results presented herein are important, because no previous studies have evaluated genetic stability using DNA molecular markers in crambe plants regenerated *in vitro*.

ISSR primers have been used to detect somaclonal variations in micropropagated plants (Verma et al., 2013) and to study genetic diversity between cultivars and germplasm (Liu et al., 2013).

Regarding genetic stability in regenerants of the family Brassicaceae, Leroy et al. (2001) used ISSR markers in *Brassica oleracea* var. botrytis and found no polymorphisms among the different regenerants originating from hypocotyl-derived somatic embryos. However, those authors also found that only six of the 224 calluses of this species exhibited genetic stability. Qin et al. (2007) established somatic embryogenesis in the *B. oleracea* var. *italica*, and after a genetic stability analysis of the regenerants using random amplified polymorphic DNA (RAPD) molecular markers, they identified 79 polymorphic bands from 20 primers, a result that, according to the authors, demonstrated the genetic stability of the regenerants. Modgil et al. (2005) reported that the long duration of culturing *in vitro* is a major cause somaclonal variation. However, in our study, culturing for 180 days was considered adequate to obtain genetically uniform regenerated plants from explant donor plants of *C. abyssinica*.

These results indicate that the apical segment is a highly reliable micropropagation method for the multiplication of genetically true plants (true-to-type). The small genetic variation detected may be due to the 180-day incubation period, successive medium changes, and the possibility of growth regulators inducing variations and/or mutations. However, for all of the primers tested, 91.2% of the bands were monomorphic, demonstrating genetic stability in the *C. abyssinica* cultivar FMS Brilhante in the present study.

This study proposes a protocol for the micropropagation of crambe using apical segments as explants, and for the maintenance of genetic stability, as assessed using ISSR markers. This standardized micropropagation protocol can be used with minimal possibility of variability being introduced during the *in vitro* process.

### Conflicts of interest

The authors declare no conflict of interest.

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