

# Indirect organogenesis and assessment of somaclonal variation in plantlets of *Vanilla planifolia* Jacks

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**Abstract** The low genetic variability of vanilla (*Vanilla planifolia* Jacks.) makes it susceptible to pests and diseases, which leads to a decrease in production. The genetic variability can be broadened by using in vitro plant tissue culture techniques. The use of indirect morphogenic routes allows increasing the percentage of somaclonal variation in regenerated plantlets. The objective of this research was to establish a protocol for indirect organogenesis in *V. planifolia* aimed at broadening the genetic base of the crop. A friable callus was induced from immature seeds grown in the dark, using MS medium supplemented with 2.27  $\mu\text{M}$  thidiazuron (TDZ). Subsequently, 6.8 shoots per callus were regenerated in MS medium supplemented with 8.88  $\mu\text{M}$  benzyladenine (BA). One hundred per cent rooting of regenerated shoots was achieved when MS medium was used with no plant growth regulators. Last, rooted plantlets were acclimatized in a greenhouse. A 91 % survival rate was observed. Molecular analyses on regenerated plantlets revealed the existence of a 71.66 % genetic polymorphism. Furthermore, morphological variation was confirmed by the presence of variegated individuals in regenerated plantlets. The proposed protocol can be useful in subsequent vanilla genetic improvement works.

**Keywords** In vitro · Polymorphism · Genetic variability · ISSR · Callus

## Introduction

Vanilla (*Vanilla planifolia* Jacks.) is a native orchid distributed in tropical and subtropical regions of the Americas (Soto-Arenas 2003). Vanilla culture is economically important because of vanillin, an organic compound highly appreciated in the food and cosmetic industry, extracted from fruits (Kalimuthu et al. 2006). In Mexico, this culture faces serious problems associated with premature fruit shedding (Castro-Bobadilla et al. 2011) and susceptibility to attack by fungal pathogens such as *Fusarium oxysporum* (Hernández-Hernández 2011).

Given its high economic value, the development of genetic improvement programs in this crop is essential to obtain highly productive disease-resistant genotypes for commercialization (Retheesh and Bhat 2011). However, the success of any genetic improvement program requires having a broad genetic base, which is limited in vanilla (Minoo et al. 2006; Retheesh and Bhat 2011).

Separately, spontaneous mutations occur at an extremely low frequency in natural populations; therefore, a promising alternative for broadening the genetic basis in *V. planifolia* is the use of in vitro plant tissue culture (PTC) to produce somaclonal variation (Cardone et al. 2010). This can achieve a rapid increase in the genetic variability of cultivated species (Lestari 2006; Mahlanza et al. 2013). The induction of morphogenetic processes involves the dedifferentiation and redifferentiation of plant somatic cells (Cardone et al. 2010; Grafi and Barak 2014), which in somaclonal variation (Larkin and Scowcroft 1981; Kaepler et al. 2000). This variation may increase in micropropagated plants through indirect morphogenic routes, because the callus cultures involves cell adaptation to the conditions of in vitro culturing causing genetic and epigenetic changes (Kaepler et al. 2000; Grafi and Barak

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2014). Friable callus obtained through indirect organogenesis, can be used to establish cell cultures for other biotechnological purposes.

Somaclonal variation can be generated through indirect organogenesis, involving both genetic (Larkin and Scowcroft 1981) and epigenetic (Kaeppler et al. 2000) changes in in vitro regenerated plants. Then, variants can be selected with desirable traits, such as resistance to diseases (Lebeda and Svábová 2010) and tolerance to various abiotic factors (Lestari 2006; Ravikumar et al. 2007).

One of the most reliable techniques for assessing genetic variation produced in vitro is inter-microsatellite sequencing through ISSR (Inter-Simple Sequence Repeat) (Pradeep et al. 2002). The ISSRs are simple, faster and reproducible method to detect polymorphic loci present in nuclear DNA and organellar DNA within repeats number, determinate by addition or deletion of repeat units or by point mutation so it can allow evaluating the somaclonal variation occurring in the tissue cultured plant at the genetic level (Jarne and Lagoda 1996).

Micropropagation of *V. planifolia* has been carried out both directly (Lee-Espinosa et al. 2008; Mengesha et al. 2012; Zuraida et al. 2013; Oliveira et al. 2013) and indirectly (Janarthanam and Seshadri 2008; Tan et al. 2011, 2013). However, none of these studies has evaluated the somaclonal variation potentially occurred in micropropagated plantlets.

This investigation aims to develop a protocol for indirect organogenesis resulting in high percentages of somaclonal variation in *V. planifolia* plantlets, in order to contribute to genetic improvement works on this crop.

## Materials and methods

### Callus induction

Immature capsules of *V. planifolia* (Mansa morphotype) of approximately 7 months of maturity were collected from the Papantla region, Veracruz, Mexico. Capsules were washed with tap water and liquid detergent for 10 min. Then, in preparation for planting, capsules were cleaned with 75 % ethanol four times in the laminar flow hood; afterwards, these were cut transversally and immature seeds were transferred to the growth medium using sterile scalpels, following the methodology proposed by Shin et al. (2011). Internode (0.5–1 cm in length) and leaf segments (1 × 1 cm) used as explants came from plantlets established in the *Vanilla* spp in vitro germplasm bank at the PTC laboratory of *Instituto de Biotecnología y Ecología Aplicada* (INBIOTECA) at the *Universidad Veracruzana*.

For the in vitro establishment of the plant material, the MS (Murashige and Skoog 1962) culture medium supplemented

with 50 mg L<sup>-1</sup> L-cysteine hydrochloride 1100 mg L<sup>-1</sup> ascorbic acid, and 30 g L<sup>-1</sup> sucrose was used; 2.2 g L<sup>-1</sup> Phytigel<sup>®</sup> was used as gelling agent. The pH of the medium was adjusted to 5.8 ± 0.2 and culture flasks were autoclaved at 1.5 kg cm<sup>-2</sup> and 121 °C for 15 min. The effect of two different plant growth regulators (PGRs) was evaluated separately at different concentrations: thidiazuron (TDZ) (0, 0.45, 1.13 and 2.27 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0 2.26, 4.52 and 6.78 μM).

Cultures were incubated at 25 ± 2 °C under an irradiation of 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by fluorescent lamps (60 W Osram<sup>®</sup>). The effect of photoperiod (16 h light/8 h dark) and complete darkness was evaluated. After 8 weeks of culture, the percentage of explants that formed a callus was assessed; also, the appearances of calluses were evaluated using the following scale: ++ friable calluses, + compact calluses, and – absence of callus.

### Shoot regeneration

Friable callus formed during induction were disaggregated into small pieces (0.5 g). Subsequently, these were transferred to MS medium to assess the effect of different benzyladenine (BA) concentrations (0, 6.66, 8.88 and 11.11 μM). Cultures were incubated under the conditions described above. The effect of photoperiod (16/8) and darkness was evaluated. After 8 weeks of culture, assessments were made on the percentage of calluses that produced shoots, number of shoots per callus and shoot length.

### Rooting

To induce rooting, 2-cm shoots (in length) were transferred to MS medium at half its concentration with no PGR supplementation, following the methodology by Zuraida et al. (2013). After 6 weeks of culture, the percentage of rooted shoots was evaluated.

### Acclimatization

Shoots previously rooted in vitro and measuring 8–10 cm in height were rinsed with tap water; afterwards, these were planted in a sterile substrate made of peat moss and agrolite (1:1 v/v) using 50 × 30 × 5 cm trays. Plantlets were grown in a greenhouse under the following conditions: 50 % shade; 80–95 % relative humidity; temperature between 28 and 32 °C, and were watered with tap water three times a week. The leaf fertilizer Nitrofoska<sup>®</sup> (N:P:K, 25:10:17) was applied once a week. When plants reached 30 cm high, these were transferred to a mixture of peat moss, agrolite and compost (1:1:1 v/v) in individual pots. Fungicide (1 g L<sup>-1</sup> Tiabendazol) and foliar fertilizer (Nitofoska<sup>®</sup>) was applied once a week in accordance with technical culture standards

(Hernández-Hernández 2011). After 8 weeks in the greenhouse, the survival rate of acclimatized plantlets was recorded. Finally, plantlets were planted in the field for subsequent genetic improvement studies.

### Assessment of somaclonal variation

#### Genomic DNA extraction

Total DNA was extracted by duplicate from 0.2 g of foliar tissue from 28 plants regenerated from friable calluses and two donor plants (Mansa morphotype), following the method by Stewart and Via (1993). The quality of genomic DNA was examined using agarose gel electrophoresis (1 %) and was quantified by spectrophotometry at an OD ratio of 260/280 using an Invitrogen® (Qubit 2.0) fluorometer.

#### ISSR analysis

12 ISSR primers (UBC, UBC, Biotechnology Laboratory, University of British Columbia) were tested. ISSR amplification reactions by PCR were performed in a total volume of 25  $\mu\text{L}$  containing 0.4 mM dNTPs, 2.5 mM  $\text{MgCl}_2$ , 25 pM primer, 0.5 U Taq polymerase (Bioline®) and 25 ng DNA templates. DNA amplification was performed in an AXIGEN® (Applied Biosystem, CA, USA) thermocycler. PCR conditions were: an initial denaturation at 94 °C for 1 min, subsequent denaturation at 94 °C for 30 s, annealing at  $T_m$  °C for 45 s, extension at 72 °C for 1 min, 35 cycles, and the final extension at 72 °C for 10 min.

The amplification products were separated by electrophoresis in 1.5 % agarose gel (w/v) using 1 $\times$  TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA and pH 8.4), at 100 V for 1.5 h. Subsequently, gels were stained with 1 mg  $\text{mL}^{-1}$  ethidium bromide. Gene Ruler DNA Plus™ 100 bp (Fermentas®) was used as DNA marker. Bands were visualized in a gel documentation system (GelDoc-It® Imager, UVP).

All the reactions for each ISSR primer were repeated twice only reproducible bands between replicates were used to establish a binary base, scoring 1 for presence and 0, absence. On these bases, the total number of bands, mean number of bands per primer, percentage of monomorphic *loci* and percentage of polymorphic *loci* were calculated.

#### Statistical analyzes

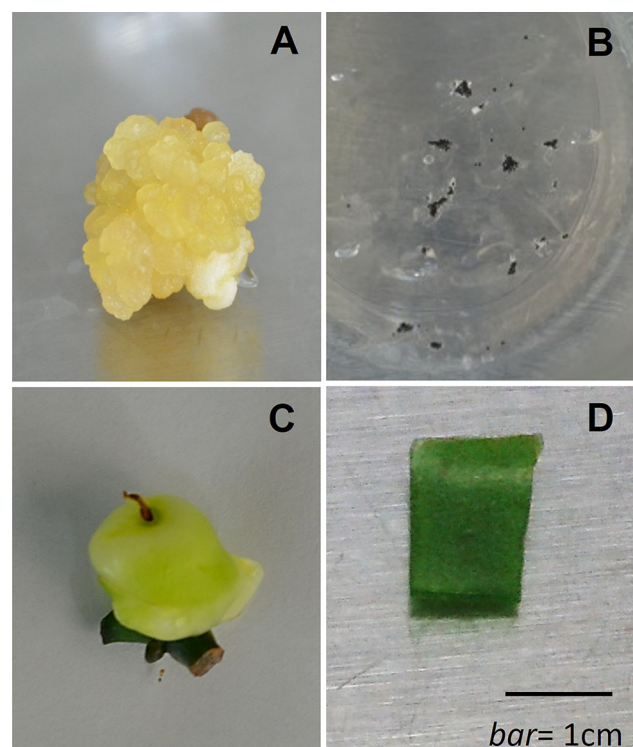
All experiments were run using a randomized design with 5 replicates each. For callus induction, a factorial analyze of variance was conducted considering the following variables: PGR type, PGR concentration and incubation conditions. Thirty explants were used per treatment. The data obtained were processed statistically with the IBM SPSS

Statistics (version 21) software. An analysis of variance (ANOVA) followed by Tukey's test ( $p \leq 0.05$ ) was performed. Percent data were arcsine transformed prior to the statistical analysis (Zar 1996).

## Results

### Callus induction

Friable callus formation was observed at 12 weeks of culture under darkness using explants in MS medium supplemented with TDZ as immature seeds (Fig. 1a). In contrast, no callus formation was observed in immature seeds were cultured under light conditions (Fig. 1b). The highest percentage of callus formation (100 %) was observed using 2.27  $\mu\text{M}$  TDZ, followed by 40 % with 0.45 and 1.13  $\mu\text{M}$  TDZ (Table 1). The callus formed using nodal segments as explants showed a compact appearance, regardless of PGR type and concentration or incubation conditions (Fig. 1c). No callus formation was achieved when foliar segments were used as explants (Fig. 1d).



**Fig. 1** Effect of different growth regulators, explant sources and incubation conditions on callus formation in *V. planifolia*. **a** Friable callus obtained using seed in media enriched with 2.27  $\mu\text{M}$  TDZ under darkness; **b** seeds in media supplemented with 2.27  $\mu\text{M}$  TDZ and 6.78  $\mu\text{M}$  2,4-D under light; and 6.78  $\mu\text{M}$  2,4-D under darkness; **c** compact callus obtained using nodal segments in media supplemented with 2.27  $\mu\text{M}$  TDZ under either light or darkness; and in media enriched with 6.78  $\mu\text{M}$  2,4-D under photoperiod; **d** foliar segments, all treatments

**Table 1** Effect of different growth regulators, explant sources and incubation conditions for callus induction in *Vanilla planifolia* at 12 weeks of culture

Explant	PGR	Concentration ( $\mu\text{M}$ )	% callus-forming explants	Callus type	% Callus-forming explants	Callus type
			Photoperiod	Darkness		
Seeds	TDZ	0.45	$0.0 \pm 0.0^e$	–	$100.0 \pm 0.0^{a***}$	++
		1.13	$0.0 \pm 0.0^e$	–	$40.0 \pm 2.1^{de***}$	++
		2.27	$0.0 \pm 0.0^e$	–	$40.0 \pm 2.1^{de***}$	++
	2,4-D	2.26	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		4.52	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		6.78	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
Leaf segments	TDZ	0.45	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		1.13	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		2.27	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
	2,4-D	2.26	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		4.52	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
		6.78	$0.0 \pm 0.0^e$	–	$0.0 \pm 0.0^e$	–
Internode segments	TDZ	0.45	$40.0 \pm 1.5^{de}$	+	$40.0 \pm 1.5^{de}$	+
		1.13	$85.0 \pm 0.5^{abc}$	+	$85.0 \pm 0.5^{abc***}$	+
		2.27	$90.0 \pm 0.5^{ab}$	+	$90.0 \pm 0.5^{ab***}$	+
	2,4-D	2.26	$50.0 \pm 1.5^{cde}$	+	$0.0 \pm 0.0^e$	–
		4.52	$60.0 \pm 1.3^{cde}$	+	$0.0 \pm 0.0^e$	–
		6.78	$75.0 \pm 1.4^{bcd}$	+	$0.0 \pm 0.0^e$	–

Numbers represent the mean  $\pm$  SE (standard error). Means with different letters are significantly different (Tukey,  $p \leq 0.05$ ). Appearance of callus; + = compact callus; ++ = friable callus; – = absence of callus

\*\*\* Significance in factorial analyzes

The use of TDZ and seeds was found to promote the formation of friable calluses in *V. planifolia*. The percentage of callus formation increased directly with PGR concentration (Table 1). Similarly, it increased when 2,4-D was used (Table 1), but these calluses were characterized by a compact appearance (Fig. 1c). Photoperiod favored the formation of compact calluses, regardless of the type of PGR used (TDZ or 2,4-D) (Fig. 1c). Under dark conditions, the type of callus obtained depended on the types of explant and PGR used: friable calluses were obtained only with seeds and TDZ (Table 1).

The factorial analyze of variance revealed significant differences of the interaction between seeds, darkness and TDZ concentration ( $F_{29, 1459} = 16.17$ ;  $p < 0.05$ ). Likewise, significant differences were found regarding the interaction between the variables: internode, darkness and 2,4-D concentration of ( $F_{29, 1459} = 22.35$ ;  $p < 0.05$ ) in callus induction in vanilla (Table 1).

### Shoot regeneration

At 8 weeks of culture, shoots development from friable calluses was promoted only under light conditions and when the medium was supplemented with  $8.88 \mu\text{M}$  BA

(6.8 shoots/explant) (Fig. 2a). In the dark, this same BA concentration ( $8.88 \mu\text{M}$ ) led only to an increase in size of the initial callus (Fig. 2b). In non-supplemented medium (without PGRs), no response was observed to either photoperiod or darkness (Fig. 2c). Shoot preforming was observed after 4 weeks of culture (Fig. 2d). Some regenerated shoots displayed a variegated leaves (Fig. 2e).

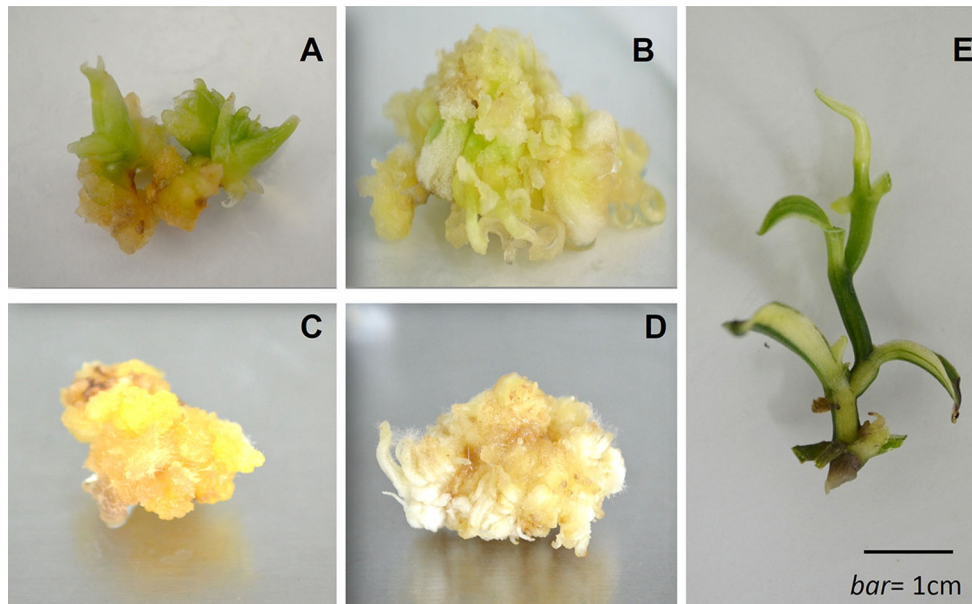
### Rooting and acclimatization

Root formation and shoot elongation occurred at 6 weeks of culture. One hundred per cent of rooted shoots was achieved following the methodology by Zuraida et al. (2013). In the acclimatization process, a 91 % survival rate was observed 8 weeks after the in vitro transfer of *V. planifolia* plantlets to the greenhouse (Fig. 3a, b). Acclimatized plantlets were finally transplanted to the field for future assessments (Fig. 3c).

### ISSR analysis

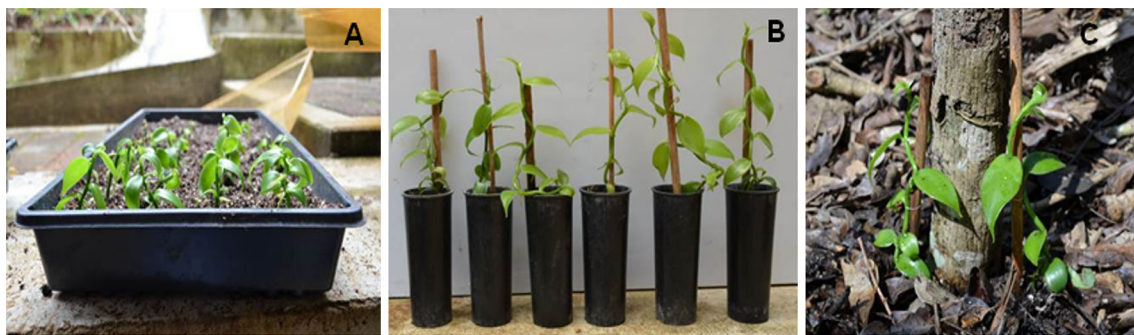
Of a total of 12, only six primers, namely 809, IS-05, IS-06, IS-15, IS-17 and IS-19, produced clear and well-defined bands; these were selected for ISSR analysis (Table 2).





**Fig. 2** Indirect organogenesis obtained from friable callus of *V. planifolia*. **a** Callus grown under photoperiod with media supplemented with 8.88 μM BA; **b** callus grown under darkness with media

supplemented with 8.88 μM BA; **c** callus grown under darkness and photoperiod in non-supplemented media; **d** shoot preformation from callus grown under photoperiod; **e** variegated regenerated plants



**Fig. 3** In-vitro regenerated *V. planifolia* plants. **a** Recently transferred plants; **b** vanilla plantlets after 8 weeks of acclimatization; **c** vanilla plants sown in the field

**Table 2** List of ISSR primers

Name	Sequence*	Ann.
809	5'-AGAGAGAGAGAGAGAGG-3'	52
818	5'-CACACACACACACACAG-3'	52
827	5'-ACACACACACACACACG -3'	52
IS-05	5'-GACAGACAGACAGACARG-3'	55
IS-06	5'-GACAGACAGACAGACARY-3'	55
IS-14	5'-GACAGACAGACAGACA-3'	40
IS-15	5'-GACAGACAGACARG-3'	45
IS-16	5'-YRGACAGACAGACA-3'	40
IS-17	5'-GACACGACAC-3'	35
IS-18	5'-ACTGACTGACTG-3'	40
IS-19	5'-ACTGACTGACTG-3'	45
IS-20	5'-YR ACTGACTGACTG-3'	45

\* Y:C/T; R:A/G

The six primers selected produced a total of 60 discrete bands ranging 112–1260 bp in size (Table 3). The number of bands per primer ranged between 7 and 14, with an average of 10 bands per primer (Table 3). Of the 60 bands produced from the 28 regenerated plantlets and two samples of donor plants (Mansa morphotype), 43 bands were polymorphic (71.66 %) and 17 were monomorphic bands (28.34 %). Figure 4 shows ISSR band profiles from 28 regenerated plantlets and two samples of donor plants using primer IS-19.

All primers produced polymorphic bands. The primer that produced more polymorphic bands (13 bands) was IS-19, followed by IS-17 with 9 polymorphic bands. On the other hand, primers IS-809 and IS-05 produced only 6 polymorphic bands (Table 3). Primer IS-19 produced a

**Table 3** Details on ISSR analysis of micropropagated plantlets by indirect and direct organogenesis using six primers

Name	Total amplified bands	No. polymorphic bands	% Polymorphic bands	Size (bp)
809	9	6	66.6	235–1210
IS-05	8	6	75.0	340–884
IS-06	10	5	50.0	380–1080
IS-15	7	5	71.4	320–1260
IS-17	14	9	64.2	120–670
IS-19	12	12	100	176–690
Total	60	43	71.66	120–1260



**Fig. 4** Pattern of ISSR bands in the *Vanilla planifolia* stock plant and in specimens regenerated by indirect organogenesis, amplified with primer IS-19. M = Molecular weight marker, lines 1–28 correspond

to indirect organogenesis (IO) micropropagated plants; lines 29–30, samples of donor plants

specific band (176 bp) (Fig. 4) in samples 13 and 14, corresponding to the variegated type analyzed (Fig. 2e).

## Discussion

This study establishes an indirect organogenesis protocol involving the induction and regeneration of friable calluses in *V. planifolia*, which resulted in high percentages of somaclonal variation. Unlike the study by Palama et al. (2010), this work achieved the production of a friable callus, useful for establishing cell suspensions for various biotechnological purposes. An example would be the generation by in vitro selection of cell lines that are tolerant and/or resistant to a number of biotic and abiotic factors. On the other hand, our results agree by inducing a compact callus using internode -leaf segments in MS medium supplemented with 2,4-D under photoperiod conditions (Janarthanam and Seshadri 2008; Tan et al. 2011, 2013).

As regards shoot regeneration, it was confirmed that BA is among the cytokinins most frequently used for in vitro shoot regeneration in *V. planifolia* (Janarthanam and Seshadri 2008; Lee-Espinosa et al. 2008; Oliveira et al. 2013; Tan et al. 2011, 2013; Zuraida et al. 2013). In our work, the two incubation conditions (photoperiod and darkness) led to different morphogenetic responses as to shoot regeneration. Similar studies have shown the importance of incubation conditions for the in vitro culture of various plant species (Jo et al. 2008; Ahmad et al. 2014).

With respect to the number of shoots, our research produced 6.8 shoots per explant (callus) using 8.88  $\mu$ M BA. These results differ from some works on shoot regeneration from calluses; some studies have achieved 14 shoots per callus (Janarthanam and Seshadri 2008), 10.3 shoots per callus (Tan et al. 2013) and 4.2 shoots per callus (Tan et al. 2011). However, BA concentrations used in these studies are higher than those used in our study. Furthermore, these shoot numbers were obtained from

compact calluses, and in some cases BA was used in combination with another PGR.

Research work has been conducted on direct organogenesis in *V. planifolia*, in which very high numbers of shoots have been produced using BA (18.5 shoots per explant) (Lee-Espinosa et al. 2008); other authors (Zuraida et al. 2013; Oliveira et al. 2013) obtained a smaller number of shoots per explant (4 and 6.06 shoots, respectively). As shown, the number of shoots produced depends on the type and concentration of PGRs, morphogenetic route, incubation conditions, and explant type and size.

One hundred per cent rooting was achieved by using MS medium with no PGRs, which is consistent with previous investigations on this species (Kalimuthu et al. 2006; Abebe et al. 2009; Oliveira et al. 2013; Zuraida et al. 2013). Similar to this study, some authors report that rooting of various plant species, particularly herbaceous plants, can be achieved by using low auxin levels, or simply in a basic medium with no growth regulators added (Gurel et al. 2011). This helps to reduce costs in the micropropagation process of various plant species.

Regarding the in vitro acclimatization of *V. planifolia* plantlets, our findings are consistent with survival rates from 70 to 95 % reported for this species by several authors (Tan et al. 2011, 2013; Zuraida et al. 2013). These results contrast with those observed for some plant species, where a high mortality has occurred during the acclimatization process due to stomatal dysfunction, weak root systems and/or poor cuticle development (Mathur et al. 2008). Therefore, the protocol established in this study also ensures a rapid and inexpensive rooting, as well as an adequate survival rate of this commercially important species.

Unlike studies on direct and indirect organogenesis in *V. planifolia*, this investigation assessed somaclonal variation in regenerated plantlets. Our findings confirm the usefulness of ISSR markers in the analysis of somaclonal variation of micropropagated plantlets, similar to works on crops such as rye (*Secale cereale* L.) (Linacero et al. 2011), hydrangea (*Hydrangea macrophylla*) (Liu et al. 2011) and lilies (*Lilium orientalis*) (Liu and Yang 2012; Yin et al. 2013). A relatively high percentage (71.66 %) of genetic polymorphism was detected in *V. planifolia* plantlets regenerated through indirect organogenesis. Likewise, the presence of some variegated plants was observed, coinciding with findings reported elsewhere (Koh and Davies 2001; Zhao et al. 2012). The protocol established here may be of interest to broaden the limited genetic base of the crop studied (Minoos et al. 2006; Rethesh and Bhat 2011). This preliminary study can open up new perspectives for implementing a biotechnological genetic improvement program for this species of growing commercial importance.

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**Authors contribution** LGIA conceived and designed research. MARM conducted experiments, analyzed and reviewed the statistical analysis. MARM and LGIA wrote the manuscript. All authors read and approved the manuscript.

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