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Original Article

Evaluation of Genetic Stability and Effects of Plant Growth Regulators for *in vitro* Propagation of Underutilized *Vitis amurensis* 'Cheongsan'

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Abstract

Vitis amurensis 'Cheongsan' is a plant with high economic value in both medical and agricultural applications. However, its utilization has been restricted owing to difficulties encountered when applying traditional mass propagation methods, requiring instead application of *in vitro* propagation methods for their mass scale production. Hence, this study was conducted to find the optimal plant growth regulators for shoot multiplication and root induction during *in vitro* propagation. Among the three cytokinins used at multiple concentrations for culture initiation and shoot multiplication, the most positive response was found with MS medium containing 5.0 μM 6-benzyladenine (BA), compared to more modest responses from other types of cytokinin, such as kinetin (KIN) and thidiazuron (TDZ). For root induction, medium supplemented with α-naphthaleneacetic acid (NAA) produced a callus and inhibited shoot growth in explants, whereas indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) did not create any significant problems, but did display differences in root induction efficiencies. Generally, root induction responses with IBA were better that those with IAA. The maximum rooting rates were observed without callus formation and no shoot growth inhibition from explants grown on media supplemented with 0.67 μM IBA. Further, inter-simple sequence repeat (ISSR) analyses revealed that micropropagated plantlets generated in medium supplemented with 5.0 μM BA and 0.67 μM IBA did not lead to genetic variation. Therefore, the application of the *in vitro* propagation method developed in this study could be used on a commercial scale and will offer opportunities to strengthen the industrial use of *V. amurensis* 'Cheongsan'.

Keywords: auxin; cytokinin; ISSR marker; somaclonal variation

Introduction

Vitis amurensis belongs to the family Vitaceae. It has been regarded as an important medical crop and has long been used in various ethno-medical systems because it contains an abundance of resveratrol and anthocyanin, which have excellent anti-cancer, anti-oxidation, and anti-inflammatory effects (Jang *et al*., 2007; Bak *et al*., 2016; Chen *et al*., 2018). In addition, it has received significant attention as a very useful breeding material, improving resistance to disease and the freezing tolerance of existing commercial grape varieties (Fennell, 2004; Chai *et al*., 2019; Fu *et al*., 2019). However, it has not been widely utilized for commercial purposes because of its substantially lower fruit quality and uneven productivity compared to commercial grape cultivars (Park *et al*., 2005a).

In order to complement these problems, some breeders have tried to improve the poor inherited characteristics of *V. amurensis* and enhance its direct use (Park *et al*., 2005b; Song *et al*., 2008). Finally, we developed *V. amurensis* 'Cheongsan' by crossing our strain with a Korean native *V. amurensis* possessing superior traits. It not only preserves all the important traits of *V. amurensis*, but also has much better fruit quality and very stable productivity (Park *et al*., 2017). Its value as a table grape and a wine grape in Korea is increasing because it has a distinguished fruit quality as an

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edible crop and properties beneficial to production of high quality wine (Heo *et al*., 2016). For this reason, the demand for *V. amurensis* 'Cheongsan' is increasing in Korea.

Grapevines are usually proliferated by stem cutting (Alley, 1980), but stems cut from *V. amurensis* do not produce roots easily (Guo *et al*., 1987). Hence, it is difficult to quickly supplying an adequate number of saplings to farmers using conventional methods of propagation, which prohibits wide commercial use of *V. amurensis* 'Cheongsan'. An alternative method for plants with these kinds of problems is an *in vitro* plant propagation method, which has been applied successfully to other systems (Hassan and Zayed, 2018). Other studies have demonstrated the merits of this approach, such as high propagation efficiencies produced through the application of plant growth regulators (PGRs) and avoidance of seasonal and space barriers for propagation work, compared to conventional propagation methods in many economically important crops (Lazo-Javalera *et al*., 2016; Mozafan *et al*., 2017; Toma, 2018). However, several researchers have indicated that the efficiency of *in vitro* propagation for *Vitis* spp. can differ significantly based on the interactions between the type and concentration of applied PGRs and the species or genotype of the crop (Aazami, 2010; Alzubi *et al*., 2012). *In vitro* propagation methods mainly consist of two steps: shoot multiplication and rooting. It is well known that shoot proliferation and rooting efficiency in *Vitis* spp. are affected by the type and concentration of cytokinin and auxin (Gray and Benton, 1991; Skiada *et al*., 2010). The efficiency of *in vitro* propagation can differ based on the genetic characteristics of the species, making it important to test their effects and to select the optimal PGR conditions.

Apart from this issue, some reports have shown that *in vitro* plant propagation methods could lead to somaclonal mutations. *V. amurensis* is a perennial crop and important genetic variations resulting from *in vitro* propagation may only be seen at maturity and fruiting. For this reason, early detection of genetic variations in *in vitro* raised plants is also important. However, examinations of morphological and phenological traits do not lead to precise determinations of clonal homogeneity. Hence, ISSR markers were chosen to screen the genetic stability of *V. amurensis* 'Cheongsan' in this study, as the assays are easily performed and highly reliable and the utilization of ISSR primers in evaluating genetic stability is well documented in several *Vitis* spp. (Nookaraju and Agrawal, 2012; Motha *et al*., 2017).

In order to assess the large-scale commercial use of *V. amurensis* 'Cheongsan', this study has investigated the optimal PGR composition for mass proliferation by *in vitro* culture and assessed the genetic stability of *in vitro* raised plants using ISSR markers.

Materials and Methods

Culture establishment

A total of 50 plants healthy greenhouse-grown 1-year-old potted *V. amurensis* 'Cheongsan' were used as source materials. For *in vitro* culture establishment, nodal explants with an axillary bud of a 2 cm long were used. They were collected from 3 to 6 node positions from the tip and downwards from young and actively growing shoots. The nodal segments were washed in running tap for 10 min, and then surface sterilization was carried out under aseptic conditions. They were immersed in 70% ethanol for 1 min, followed by 2% (w/v) NaOCl for 10 min, and then rinsed with sterile distilled water 5 times. For culture initiation, each nodal segment was placed in a Pyrex glass culture tube (22 mm × 200 mm) containing 1× Murashige and Skoog (MS) medium supplemented with various cytokinins: 6-benzyladenine (BA), kinetin (KIN) or thidiazuron (TDZ) at 1, 2.5, 5, 7.5, and 10 μM, supplemented with sucrose at 30 g/L^{-1}, and solidified with Difco-Bacto agar at 8 g/L^{-1} separately. After 4 weeks of initial culture, induction of shoot organogenesis was investigated for each treatment. Each treatment had a total of 30 explants. Cultures were incubated at a temperature of 25 ± 1 °C under 1,500-lux light intensity with 16 h of day light per photoperiod.

Effects of plant growth regulators for shoot proliferation

After 4 weeks of initial culture, micro cuttings from the first-generation *in vitro* shoots were sub cultured in culture vessels (85 mm × 110 mm) with the same PGR composition except for KIN and incubated under the culture conditions described above to determine the optimum PGR conditions for multiple shoot proliferation. The number of shoots and nodes per explant were recorded along with the length of the main shoot after the 30 days of subculture. Each treatment for shoot multiplication consisted of four (4) replications each with six (6) explants.

Effects of plant growth regulators for root induction and acclimatization

For evaluation of PGR effects on root induction, elongated shoots derived from inoculated nodal segments were additionally sub cultured on the medium supplemented with 5 μM BA until sufficient plant material was collected. When the experiment was ready, micro cuttings greater than 0.7 cm in length were prepared and moved to culture vessels supplied with different kinds of auxin or plant growth regulator free media. To determine optimum PGR conditions, a total of three types of auxin were tested: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) at 0.33, 0.67, 1.0, 2.5, and 5.0 μM. The conditions for the *in vitro* cultures were the same as those used for the shoot proliferation. The following data were assessed 30 days after the *in vitro* rooting treatments: rooting percentage, callusing percentage, total number of roots, main root length, and shoot length. Each treatment for root induction also consisted of four (4) replicates each with 6 explants.

Rooted plantlets were planted into a commercial substrate mix (Nongwoo Bio, Suwon, Korea) contained in plastic sundae cups with lids, placed in a Microclimate chamber at 25 °C, and hardened by gradually lowering the humidity over 4 weeks. Hardened-off plants were transferred into potting medium in 1 L plastic pots and maintained in the glasshouse of Gangneung-Wonju National University in Korea.

Statistical analysis for shoot proliferation and root induction

Statistical analyses of the data were carried out with the SPSS 25.0 software program for Windows (SPSS Inc., Chicago, USA). An analysis of variance of treatment was performed to determine the effects of the treatments. When the treatment effects were statistically significantly ($P<0.05$), means were compared with Duncan's multiple range test.

Analysis of genetic stability by ISSR marker

A set of 10 *in vitro* raised plants derived from explants using 5.0 µM BA for shoot multiplication and 0.67 µM IBA for root induction were randomly selected for evaluation of genetic stability by ISSR markers. Total genomic DNA was isolated from young leaf tissue of each *in vitro* derived plant by using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. A total of 15 ISSR primers were initially screened. Each reaction was prepared by using an Accupower premix (Bioneer, Daejeon, Korea) in a final volume of 20 µL containing 10 pM of each primer and 2 µL of genomic DNA template.

PCR amplifications were performed in a Dice® Touch (TaKaRa, Otsu, Japan) thermal cycler as follows: initial denaturing at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 48, 50, or 55 °C for 45 sec, amplification at 72 °C for 2 min, and a final extension step of 7 min at 72 °C. A 4-µL aliquot of the reaction mixture was resolved by electrophoresis on a 1.5% agarose gel in 1X TAE buffer stained with GreenStarTM (Bioneer, Daejeon, Korea). Gels were visualized under UV light and photographed using a gel documentation system. Only consistently produced and well resolved fragments, in the size range of 100 bp to 2,500 bp, were considered and scored as present or absent for ISSR markers in each sample. The genetic associations were assessed by calculating the Jaccard's similarity coefficient, the similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA), and a dendrogram was generated by using NTSYS-PC Ver. 2.1 software.

Results and Discussion

Evaluation of hormonal effects for culture initiation

The initial response of nodal segments incubated on different kind of cytokinin over a period of 30 days in culture is shown in Table 1. The sprouting of buds could be observed within two weeks of nodal segment incubation. The proportion of regeneration in nodal segments depended on the type and concentration of cytokinin used for the study, ranging from 46.7 ~ 100% (Table 1). Culture medium without cytokinins failed to regenerate or to induce shoot and bud formation from nodal segments. However, nodal segments grown in media containing cytokinins were regenerated with callus formation after 10 days of culture and the generation of new axillary buds was frequently observed after 3 weeks of culture. Plants cultured in presence of BA and TDZ were found to have an acceptable regeneration rate and an adequate number of nodes and shoots. Among all the culture media examined, media containing BA produced the best response in terms of shoot and node formation. There was no significant difference in the regeneration rate among the different concentrations of BA supplement in the culture media, but number of shoots and nodes per nodal segment increased as BA concentration increased. Although the initial culture efficiency in media supplied with TDZ was slightly lower compared to that of BA, TDZ also had a positive effect on the number of axillary buds and shoots compared to cytokinin free medium. However, media treated with KIN had a much lower number of nodes and shoots, comparable to other media, with no difference between KIN and cytokinin free treatment. In *Vitis* spp. and other important woody crops, some researchers have also shown that KIN does not provide good results compared to BA and TDZ

Table 1. Effect of cytokinin type and concentration of plant growth regulators on culture establishment in *Vitis amurensis* 'Cheongsan'

Plant growth regulators / Concentrations	Bud induction rate / nodal segment (%)	No. of shoots / nodal segment	No. of nodes / nodal segment	Length of main shoot (cm)
None	60.0^{b}	0.60^{d}	2.27^{d}	1.15^{c}
BA 1.0 µM	90.0^{a}	1.07^{bc}	3.83^{ab}	2.03^{ab}
BA 2.5 µM	96.7^{a}	1.17^{abc}	4.10^{a}	2.18^{ab}
BA 5.0 µM	93.3^{a}	1.27^{ab}	4.37^{a}	2.12^{ab}
BA 7.5 µM	96.7^{a}	1.33^{ab}	4.50^{a}	1.87^{ab}
BA 10.0 µM	96.7^{a}	1.40^{a}	4.63^{a}	1.77^{abc}
KIN 1.0 µM	50.0^{b}	0.50^{d}	2.23^{d}	1.62^{bc}
KIN 2.5 µM	53.3^{b}	0.67^{d}	2.90^{bcd}	2.08^{ab}
KIN 5.0 µM	60.0^{b}	0.63^{d}	2.73^{cd}	2.31^{ab}
KIN 7.5 µM	60.0^{b}	0.60^{d}	2.53^{d}	1.98^{ab}
KIN 10.0 µM	46.7^{b}	0.47^{d}	2.00^{d}	1.58^{bc}
TDZ 1.0 µM	86.7^{a}	0.93^{c}	3.67^{abc}	1.94^{ab}
TDZ 2.5 µM	93.3^{a}	1.07^{bc}	4.17^{a}	2.36^{ab}
TDZ 5.0 µM	100.0^{a}	1.17^{abc}	4.43^{a}	2.48^{a}
TDZ 7.5 µM	93.3^{a}	1.07^{bc}	4.03^{a}	2.20^{ab}
TDZ 10.0 µM	90.0^{a}	1.03^{bc}	4.10^{a}	2.29^{ab}

Note: Means followed by the same letter within each column are not significantly different at P 0.05.

(Alizadeh *et al*., 2012; Jamwal *et al*., 2013). Hence, the application of KIN at the lowest concentration used for this study appeared to have no positive effect for regeneration or for new bud induction in *V. amurensis* 'Cheongsan'.

Evaluation of hormonal effects for shoot proliferation

BA and TDZ were chosen for further study in order to determine the optimal cytokinin and most effective concentration for shoot multiplication, as treatment with these agents had positive effects during the initial stages of culture. Subculture responses of explants prepared from the initial cultures also differed with the type and concentration of cytokinin (Table 2). Overall, media containing BA had better subculture responses, but the difference between BA and TDZ was small. From all bud-induced explants, at least one shoot had emerged with small callus formation soon after bud break, but the degree of formation of additional shoots and nodes was significantly different depending on the type and concentration of cytokinin examined. However, media supplemented with either cytokinin produced more shoots, nodes, regardless of concentration, compared to cytokinin-free media; thus, shoot, and node induction in the subculture process appeared to be a function of the cytokinin.

TDZ had a very positive effect on main shoot development and no negative symptoms regarding the shoot and node formation were observed. Subculture responses, determined by the shoot induction in media containing TDZ, were measured from 83.3 to 91.7% and the number of shoots, the number of nodes, and the length of the main shoot ranged from 1.08 to 1.58, from 4.08 to 5.42, and from 1.39 to 1.74, respectively. Other criteria tended to respond better as the concentration of TDZ increased to 7.5 µM, then decreased at higher concentrations. It has been frequently reported that TDZ applications for shoot induction cause fascination and inhibition of shoot elongation in *Vitis* spp. as a side effect of high cytokinin activity with TDZ, but we could not investigate these types of problems. However, others have reported positive effects of TDZ on multiple shoot formation, without any negative phenomenon, in different wild plant species (Sivanesan *et al*., 2011; Grabkowska *et al*., 2014; Siddique *et al*., 2015). Hence, these results suggest that application of TDZ for *in vitro* propagation can also be useful in *V. amurensis* 'Cheongsan', although TDZ and BA provided similar positive effects.

Subculture responses in media containing BA were good during the initial culture period and remained very stable at all concentrations. In addition, the number of shoots and nodes improved even at relatively low concentrations. Among all hormonal conditions examined for this study, the highest number of shoot and nodes per explant were observed with media containing 5.0 µM BA, which produced 1.96 shoots and 6.42 nodes per explant. These values were 1.24- and 1.18-fold greater than TDZ at 7.5 µM. The optimal type and concentration of cytokinin for *in vitro* propagation varied considerably among different plant species (Fan *et al*., 2017; Kefayeti *et al*., 2019), but earlier work has reported the positive stimulating effects of BA on multiple shoot formation for several plants (Modgil and Thakur, 2015; Khatun, 2017). In *Vitis* spp., the optimum BA concentration for shoot multiplication was found to range from 2.22 to 11.1 µM, while the number of shoots and nodes decreased in explants at supra-optimal BA concentrations, resulting in complete suppression of bud break or more compact callus formations that inhibited shoot growth (Mozafari *et al*., 2017; Yildirim and Ozdemir, 2018). Other studies have reported that of the five BA concentrations examined, 5.0 µM BA showed the best results in the number of shoots and nodes per explant, while concentrations higher than 5.0 µM BA caused these numbers to decrease. More importantly, the initial culture efficiency at 5.0 µM BA was very acceptable and the shoots generated from these initial cultures, fed into a subculture application using 5.0 µM BA, had more uniform size and good leaf appearance with a green color. These results strongly indicate that 5.0 µM BA is appropriate for sustainable *in vitro* propagation of *V. amurensis* 'Cheongsan'.

Table 2. Effect of cytokinin type and concentration of plant growth regulators on shoot multiplication in *Vitis amurensis* 'Cheongsan'

Plant growth regulators / Concentrations	Culture response (%)	No. of shoots/explant inoculated (no.)	No. of nodes /explant inoculated (no.)	Length of main shoot (cm)
None	33.3^{b}	0.38^{e}	1.28^{c}	0.47^{b}
BA 1 µM	95.8^{a}	1.38^{abcd}	4.75^{ab}	1.26^{ab}
BA 2.5 µM	87.5^{a}	1.54^{abcd}	5.46^{ab}	1.33^{ab}
BA 5 µM	91.7^{a}	1.96^{a}	6.42^{a}	1.57^{ab}
BA 7.5 µM	87.5^{a}	1.79^{abc}	5.96^{ab}	1.31^{ab}
BA 10 µM	83.3^{a}	1.88^{ab}	6.00^{ab}	1.20^{ab}
TDZ 1 µM	91.7^{a}	1.08^{d}	4.08^{b}	1.45^{ab}
TDZ 2.5 µM	83.3^{a}	1.17^{bc}	4.42^{ab}	1.58^{ab}
TDZ 5 µM	83.3^{a}	1.29^{bc}	4.92^{ab}	1.4^{ab}
TDZ 7.5 µM	87.5^{a}	1.58^{abcd}	5.42^{ab}	1.74^{a}
TDZ 10 µM	87.5^{a}	1.42^{abcd}	5.04^{ab}	1.50^{ab}

Note: Means followed by the same letter within each column are not significantly different at P 0.05.

Evaluation of hormonal effects for root induction

Differential responses were observed for root induction in explants as a function of the type and concentration of auxins (Table 3). A lower rate of root induction was observed when explants produced from 5.0 μM BA were cultured on media without the application of plant growth regulators. Under non-optimal conditions, the lower part of the explants became black or produced a callus within two weeks and root growth stopped after 1 cm of growth. Overall, IBA was more effective than IAA and NAA, as it had a higher rooting rate percentage and a greater number of roots. Among the three auxins examined at multiple concentrations, 0.67 μM IBA was the most effective hormone, as it showed the maximum percent rooting (87.5%) and had the second highest number of roots produced (3.25) without any callus formation. At the same level of hormone, IAA and NAA produced 2.21 roots at a rooting rate of 70.8% and 2.46 roots at a rooting rate of 66.7%, respectively (Table 1). In this experiment, the root number was enhanced by treatment with 1 to 5 μM NAA, but these treatments induced callus formation. Moreover, shoot growth was significantly inhibited at higher concentrations of NAA compared to other types of auxin. In contrast, IAA did not lead to the formation of callus or notable growth inhibition at lower concentrations, but most important criteria, including the rooting rate, were lower than those with IBA.

Several researchers have demonstrated the rooting of explants is strongly influenced in many important woody plants by the type of auxin used (Oliveira *et al*., 2016; Rajoriya *et al*., 2018). It has been well established that the optimal type of auxin hormone for root induction differs with species or genotypes. While positive results have been reported with NAA applications for root induction in several species (Azhar *et al*., 2018; Naaz *et al*., 2019), some studies have also reported toxicity and callus formation problems resulting in unsuccessful acclimatization of plantlets (De Klerk *et al*., 1997; Alosaimi *et al*., 2018). In this study, side effects, like callus formation and non-shoot induction, were also observed in *V. amurensis* 'Cheongsan' following the application of NAA and these effects became worse at higher NAA concentrations. Our negative findings concerning NAA applications to the process of root induction are similar with previous reports and might indicate a genetic property, such as sensitivity to NAA toxicity in *V. amurensis* 'Cheongsan'. In addition, IAA applications were also less effective for root induction in *V. amurensis* 'Cheongsan' compared to IBA applications. This result can be explained by the physical characteristics of IAA, which can breakdown with light exposure and in the solid agar plate media conditions used in this study, compared to IBA (Dunlap and Robacker, 1988), or the reduced interaction effect between the remaining BA, used for the shoot multiplication process, and the IAA, used for root induction, compared to IBA. Although further investigation is needed to provide a clear explanation of these observations, our results indicate that application of IBA might be more effective for the *in vitro* propagation of *V. amurensis* 'Cheongsan' in terms of stability, persistence, and toxicity protection in media when utilizing our protocol.

Good physiological conditions are absolutely critical for successful acclimatization of plantlets. In a survival test using 42 plantlets produced with 5.0 μM BA for shoot induction and 0.67 μM IBA during acclimatization (data not published), only 2 plantlets died; thus, no survival problems were found. These results suggest that the application of 5.0 μM BA and 0.67 μM IBA during shoot multiplication and root induction is very effective for *in vitro* propagation of *V. amurensis* 'Cheongsan'.

Table 3. Effect of auxin type and concentration of plant growth regulators on root induction in *Vitis amurensis* 'Cheongsan'

Plant growth regulators / Concentrations	% of rooting	% of callusing	Root number	Root length (cm)	Shoot length (cm)
None	41.7^{b}	0^{d}	0.42^{e}	0.73^{c}	0.55^{e}
IAA 0.33 μM	75.0^{a}	0^{d}	2.04^{bcd}	1.34^{a}	1.22^{abcd}
IAA 0.67 μM	70.8^{ab}	0^{d}	2.21^{bcd}	1.18^{abc}	1.36^{abcd}
IAA 1.0 μM	75.0^{a}	0^{d}	2.50^{abcd}	1.00^{abc}	1.26^{abcd}
IAA 2.5 μM	70.8^{ab}	0^{d}	2.79^{abcd}	1.08^{abc}	1.48^{abc}
IAA 5.0 μM	66.7^{ab}	0^{d}	2.54^{abcd}	0.90^{abc}	1.31^{abcd}
IBA 0.33 μM	83.3^{a}	0^{d}	2.29^{bcd}	1.05^{abc}	1.46^{abcd}
IBA 0.67 μM	87.5^{a}	0^{d}	3.25^{ab}	1.20^{abc}	1.54^{ab}
IBA 1.0 μM	79.2^{a}	12.5^{cd}	2.96^{abcd}	1.28^{ab}	1.65^{a}
IBA 2.5 μM	70.8^{ab}	8.3^{cd}	2.45^{abcd}	1.08^{abc}	1.25^{abcd}
IBA 5.0 μM	66.7^{ab}	20.8^{c}	1.92^{cd}	0.73^{c}	0.98^{cde}
NAA 0.33 μM	62.5^{ab}	54.1^{b}	1.83^{d}	0.81^{bc}	0.65^{e}
NAA 0.67 μM	66.7^{ab}	58.3^{ab}	2.46^{abcd}	1.02^{abc}	0.91^{de}
NAA 1.0 μM	70.8^{ab}	70.8^{ab}	3.00^{abcd}	1.18^{abc}	1.06^{bcd}
NAA 2.5 μM	70.8^{ab}	70.8^{ab}	3.13^{abc}	0.92^{abc}	0.00^{f}
NAA 5.0 μM	79.2^{a}	79.2^{a}	3.67^{a}	1.04^{abc}	0.00^{f}

Note: Means followed by the same letter within each column are not significantly different at P 0.05.

Table 4. List of ISSR primers used for this study and number of band classes generated

ISSR primer	Annealing temperature (°C)	No. of distinct band classes	No. of distinct band	Total number of bands amplified	% of similarity
UBC 808	50	9	9	99	100
UBC 823	50	4	4	44	100
UBC 825	55	6	6	66	100
UBC 836	50	8	8	88	100
UBC 840	50	8	8	88	100
UBC 857	55	6	6	66	100
UBC 864	48	7	7	77	100
UBC 873	50	10	10	110	100
UBC 880	50	10	10	110	100
UBC 885	50	11	11	121	100
UBC 891	55	11	11	121	100

Analysis of genetic stability reported by ISSR marker

Genetic stability was assessed among 10 *in vitro* raised plants and compared with their mother plant. A total of 15 ISSR primers were tested, out of which 11 ISSR primers were successful in amplifying the genomic DNA and giving reproducible bands, producing 90 distinct and scorable bands. The number of scorable bands varied from 100 to 2,000 bp with an average of 8.2 bands per primer. All the primers generated monomorphic bands across all the *in vitro* raised plants and the mother plant shown in Fig. 1, indicating that *in vitro* raised *V. amurensis* 'Cheongsan' preserved genetic stability without any notable morphological differences. Our result is consistent with other findings showing no genetic variations from *in vitro* raised plantlets of several species of plants (Purohit *et al*., 2016; Jena *et al*., 2018; Behera *et al*., 2019).

The presence or absence of variation during *in vitro* propagation depends on the source of the explants and the method of regeneration (Goto *et al*., 1998). Among the various *in vitro* propagation methods, the utilization of nodal segments is most widely used (Rani *et al*., 1995) because of its simplicity as an *in vitro* propagation system and its high multiplication efficiency. More importantly, plants established from adventitious buds around the axillary buds, or from other well-developed meristematic tissue of the nodal segment, had the lowest risk of genetic variation (Martins *et al*., 2004; Joshi and Dhawan, 2007). However, it has been reported that plants derived from these tissues are not always genetically identical, as the use of sub- and supra-optimal levels of plant growth hormones, especially synthetic ones, can also cause somaclonal variation (Martin and Pachathundkandi, 2006). These results indicate that the hormonal conditions applied in this study for shoot multiplication and root induction were also optimal for the maintenance of genetic stability in *V. amurensis* 'Cheongsan' during *in vitro* culture.

Conclusions

In vitro plant propagation is important to the effective production of plants that are genetically identical to the mother plant. In this study, we found that the application of 5.0 μM BA and 0.67 μM IBA can be highly effective for *V. amurensis* 'Cheongsan' plantlet production, generating a

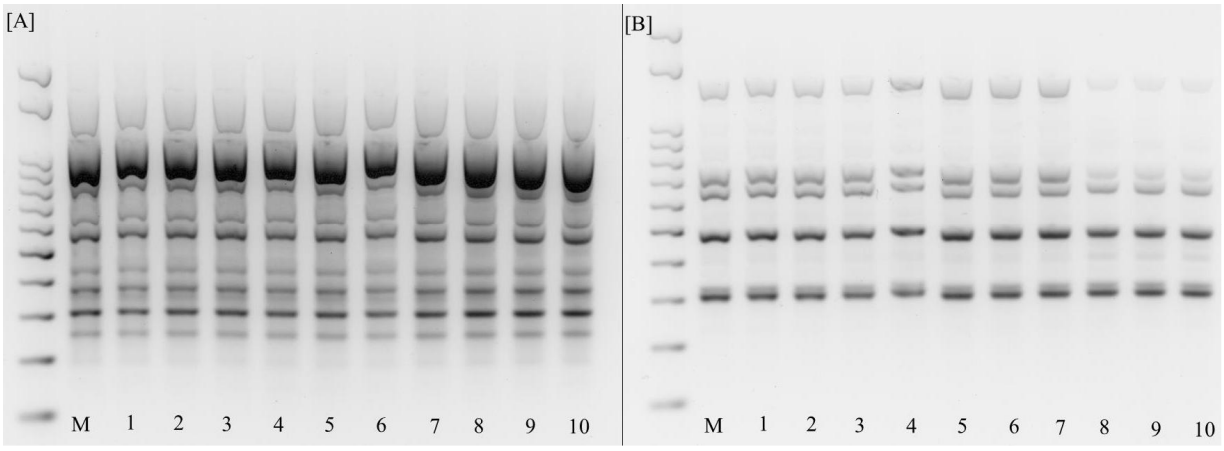

Fig. 1. ISSR profiles of *in vitro* raised *Vitis amurensis* 'Cheongsan' leaf DNA samples with UBC 808[A] and 825[B]. M: mother plant, 1-10: *In vitro* raised plants

greater number of shoots and roots compared to hormone free growth, or compared to the application of other kinds of hormones. Furthermore, no somaclonal variation was found among *in vitro* raised plants using 5.0 µM BA for the shoot induction step and 0.67 µM IBA for the root induction step. Hence, *in vitro* plant propagation by our protocol can be successfully employed to ensure genetic stability in the commercial multiplication of *V. amurensis* 'Cheongsan'.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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