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Original Research Article

Optimization of Shoot Induction, Histological Study and Genetic Stability of *in vitro* Cultured *Pisum sativum* cv. 'Sparkle'

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Abstract - An efficient shoot regeneration condition for pea cv. 'Sparkle' was developed by using optimum explant, plant growth regulator concentrations, and pretreatment of BA onto explant. The average shoot number per explant showed the highest on two kinds of shoot induction media (MSB5 media containing 2 ㎎/L BA and a combination of 2 ㎎/L BA and 1 ㎎/L TDZ) when cotyledonary node explants were cultured. Moreover, the pretreatment of explant in 200 ㎎/L BA solution was found to be more effective in shoot induction than that of non-pretreatment. By histological study, cell division and proto-meristem were formed near the surface of the sub-epidermal and epidermal cell layers of cotyledonary node in earlier than 3 days after culture. The analysis of genetic stability of regenerants by using thirteen ISSR markers showed that *in vitro* regenerated plants showed polymorphism with 8.3% compared with their mother plants.

Key words – Cotyledonary node, Genetic Stability, Histological study, Pea, Shoot Regeneration

Introduction

Pea (*Pisum sativum*) is one of important legume crops in the world. It is commonly used as a protein source for animal and human diet, and also used as a natural nitrogen source which is produced by a symbiotic bacterium in their root nodule and helpful for terrestrial ecosystem. Pea has been considered as recalcitrance for *in vitro* regeneration (Schroeder *et al*., 1993; Zhihui *et al*., 2009) and genetic modification (Aftabi *et al*., 2018; Tzitzikas *et al*., 2004). The successful *in vitro* manipulation is depended on three main factors including physiology of plant donor, *in vitro* manipulation approach, and stress physiology during plant cultivation (Benson, 2000). Moreover, genotype is an important for plant manipulation; different genotype gives the different response to regeneration efficiency (Sainger *et al*., 2015). Previous studies suggested that pretreatment of explants have effective for increasing shoot organogenesis and also essential for the early stages of transformation (Cao and Hammerschlag, 2002; Swartz *et al*., 1990).

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In order to understand the origins of the plant shoots during organogenesis it is important to carry out the histological study to determine the route of *in vitro* plant regeneration (Dibax *et al*., 2013). However, a few studies have been executed on the connection between histological and plant growth regulators variation during shoot regeneration (Wang *et al*., 2015). The observations were carried out to understand the pattern of adventitious shoot regeneration from explants. Fatima and Anis (2012) indicated that new meristem is formed closed to the exposed surface of the explants which interacts with the severed vascular bundles bringing about shoot formation. In addition, Jackson and Hobbs (1990) indicated that shoot buds of pea (*Pisum sativum*) which is produced from cotyledonary node explant were formed from superficial layers of tissues. Together, histological evaluations conducted in conjunction with media manipulation studies are means to improve culture efficiency, and can provide information to allow the application of the most appropriate strategies for plant regeneration (Woo and Wetzstein, 2008).

Maintaining genetic stability of regenerated plants *in vitro* relative to the mother plant is important to enable subtle phenotypic, cytological, biochemical, and/or molecular varia-

tions that detected (Werner *et al*., 2015). ISSR (inter-simple sequence repeats) is a highly reproducible marker system widely utilized to analyze the genetic homogeneity between mother plants and tissue cultured plants.

In the present study, we investigated the optimum condition for shoot regeneration in pea cv. 'Sparkle' using cotyledonary node explants, and histological observation was investigated to understand shoot initiation region and their changes during shoot organogenesis. And also the micro-propagated plants were evaluated for their genetic stability by employing inter simple sequence repeats (ISSR) markers.

Materials and Methods

Plant materials

For surface sterilization, seeds purchased from Korean company were soaked in 70% ethanol for 30 s - 1 min, and then dipped into 1% sodium hypochlorite solution for 15 min, followed by washing 4 times with sterile distilled water and let dry on filter paper in a laminar air flow chamber. Sterilized seeds were cultured on germination media contained 8 g/L agar. After 7 days, cotyledonary nodes (Fig. 1-A) were cultured for 3 weeks for shoot induction on MS (Murashige and Skoog, 1962) media supplemented with 2 ㎎/L BA. The explants were cultured on plant maintenance MS media for 2 weeks. Three samples of young leaflets from the mother plant and 10 samples of *in vitro* regenerated shoots were collected for DNA extraction.

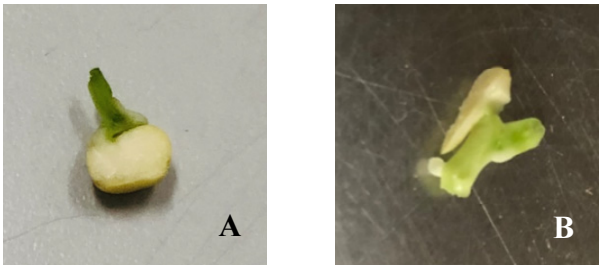

Fig. 1. Two kinds of explants used throughout this experiment. A: explant with cotyledonary nodes, B: half-split cotyledon originated from germinated *Pisum sativum* cv. 'Sparkle'.

Media preparation, culture conditions and shoot regeneration frequency

According to Raveendar *et al*. (2009), MS media supplemented with B5 vitamin were used as basal media for organogenesis. The MS medium was reinforced with 30 g/L sucrose, 8 g/L agar and the pH of the medium was regulated to 5.7 ± 0.5 after adding plant growth regulators. The medium was autoclaved at 121℃, for 20 min and poured in a culture plates. All the cultures media were kept in a sterilized culture room at 26 ± 2℃ under 16-h photoperiod provided by cool-white fluorescent lamps at 25 μmol/㎡/sec.

To estimate the optimal shooting condition, basal media were supplemented with single of benzyladenine (0, 1, 2, 4 ㎎/L BA), and combinations of benzyladenine (0, 1, 2, 4 ㎎/L BA) and thidiazuron (1, 2 ㎎/L TDZ) (Table 1).

The number of shoots and the shoot regeneration frequency were recorded after 21 days in culture. The frequency of shoot regeneration was calculated as follows:

$$\text{Shoot regeneration ratio (\%)} = \frac{\text{Number of explants regenerated into shoots}}{\text{Total number of cultured explants}} \times 100$$

Pretreatment of plant growth regulator (PGR) and selection of explant types

Two types of cotyledonary node explants were prepared by a scalpel to remove any excess hypocotyl remaining 0.5 in length (Fig. 1-A) and half of cotyledons were split off (Fig. 1-B). All of explants were dipped in sterilized 200 ㎎/L of BA solution for 1 min pretreatment and cultured on shoot induction media containing 2 ㎎/L BA. After 3 weeks, the shoot number and the shoot regeneration ratio were measured.

Histological study on shoot formation

Cotyledonary node explants were cultured in *in vitro* on the shoot induction MS media supplemented with B5 vitamin containing 2 ㎎/L BA. The cotyledonary node specimens were collected in 0, 3, 6, 9, 12, 15, 18 and 21 days. The specimens were fixed in a mixture of ethanol, formaldehyde and acetic acid for 48 hours. And then, specimens were trimmed and dehydrated by immersing the specimens in a series concentration of alcohol (70~100%) to remove the

water and formalin from the specimens. The specimens were then infiltrated and embedded into glycol methacrylate (JB-4 Embedding Kit; Poly Sciences, Warrington, PA, USA). Serial sections, 5 ㎛ thick, were cut using an ultra-microtome (MT-990 Type S, RMC Boeckeler, AZ, USA), mounted on slides, and stained by periodic acid-Schiff reaction method using hematoxylin (Sigma Co.) as a counter-strain. After rinse slides with tap water, kept the slides warm until dried on a hot plate at 35℃ for 10 minutes. After 1 week, mounted coverslip onto the section on slide glass with Permount (Fischer Chemical, Pittsburgh, PA, USA). Then, the histological changes of shoot regeneration were observed under a light microscope (Nikon E200, Nikon, Tokyo, Japan).

Genomic DNA extraction and qualification

Three young leaflets from the mother plant and 10 samples from *in vitro* regenerated shoots were collected for DNA extraction. Genomic DNA was isolated by using Qiagen commercial kit (DNeasy Plant Mini Kit, Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA content was measured with uv/vis spectrophotometer (Amersham Biosci., Ultrospec 210 pro) and diluted with sterile distilled water to adjust the final concentration to 20 ng/μL by each sample before used.

DNA amplification and ISSR analysis

The reaction mix for 20 μL reaction comprised 50 ng template DNA, 0.5 mM primer, and 10 μL of Lugen™ Sensi 2X PCR Premix (Lugen Sci Co., Ltd., ROK), adjusts total volume with sterile distilled water. PCR amplification was performed on Thermocycler (Perkin-Elmer, GeneAmp PCR System 2400) using the following program: denaturation 5 min (94℃), then 40 cycles of 45 sec at 94℃, 45 sec at 52℃ and 45 sec at 72℃, with the final 5 min extension at 72℃. The amplified PCR products were visualized and photographed using the gel imaging system (Bio-Rad, US/170-8060) after electrophoresis on a 1.5% (w/v) agarose gel containing 0.05 μL/mL of RedSafeTM Nucleic Acid Staining Solution 20,000x (Intron Biotechnology, Inc.). ISSR primers were designed and out of 20 primers tested, 13 primers were selected finally for the analysis (Table 2).

Results and Discussion

The optimizing plant growth regulators for shoot regeneration

Shoot regeneration was investigated on the media containing different concentration of BA and TDZ using the cotyledonary node explants (Table 1). Most of explants produced shoots from 76.6 to 100% on the media containing BA and a combination of BA and TDZ. MS media supplemented with BA 2 ㎎/L and combinations of BA and TDZ were very effective for high frequency shoot regeneration in 21 days. The MS media containing BA 2 ㎎/L showed the highest number of shoot followed by 2 ㎎/L BA + 1 ㎎/L TDZ and 2 ㎎/L BA + 2 ㎎/L TDZ, respectively. A differential response of shoot induction was observed from different kinds of plant growth regulator with various concentrations.

Many reports have been supported that cytokinin is the

Table 1. Shoots induction under different concentrations of plant growth regulators in 21-day

PGRs[z]		No. of explants cultured	No. of shoot per explant	Total number of shoot	Shoot regeneration frequency (%)
BA	TDZ				
0	0	30	0.20[cy]	6	25.0 (5/30)[x]
1	0	30	1.20[b]	36	76.6 (23/30)
2	0	206	3.16[a]	651	93.6 (193/206)
4	0	30	1.03[b]	31	76.6 (23/30)
2	1	65	3.06[a]	199	100.0 (65/65)
2	2	72	2.69[a]	194	94.4 (68/72)

[z]Plant growth regulators.

[y]The same letters for this column are not significantly different according to Duncan's multiple range test ($p < 0.05$).

[x]Numbers in the parentheses mean (A/B) = Number of explants regenerating shoots (A) per total number of cultured explants (B).

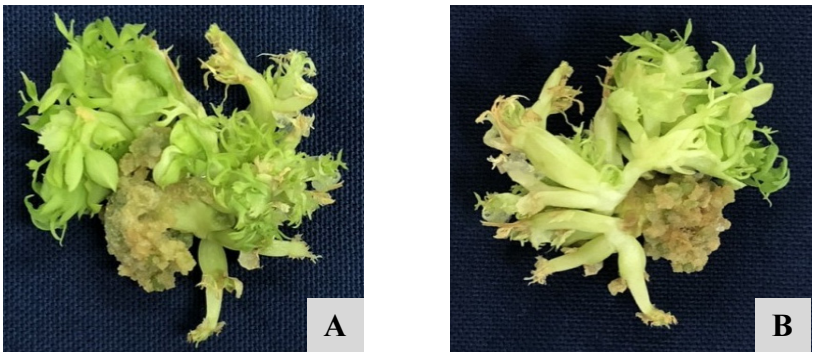

Fig. 2. Shoot regeneration from half-split cotyledon explant of pea cv. 'Sparkle'cultured on the basal MS + B5 vitamin and 2 ㎎/L BA combined with 1 ㎎/L TDZ after 45 days. A: above site, B: below site.

accelerate plant growth regulators for *in vitro* shoot multiplication (Kantayos, 2019; Kim *et al*., 2016). However, no shoot induction in the control without plant growth regulators is general. In the present study, MS media supplemented with 2 ㎎/L BA gave a marked effect on shoot regeneration. In addition, half-split cotyledon showed multiple shoot regeneration on the shoot induction media containing 2 ㎎/L BA + 1 ㎎/L TDZ in 45days (Fig. 2).

Explants cultured on the media containing a combination of auxin and cytokinin exhibited the maximum number of shoots compared with that of the media adding single PGRs (Azade *et al*., 2016; Shah *et al*., 2015; Yi *et al*., 2018). On the other hand, using a single cytokinin at the optimum concentration showed more effect on shoot regeneration than that of combination treatment (Bohmer *et al*., 1995). Veltcheva *et al*. (2003) indicated that most legume species require higher concentrations of cytokinins than do other plant families. Moreover, there are differences in the regeneration capacity between various genotype, type and size of explants, type of cytokinin, and duration of subculture and phenol concentration which is released surrounding plant during cultivation (Raveendar *et al*. 2009; Yancheva *et al*., 1999). Therefore, *in vitro* shoot regeneration via direct organogenesis was investigated by using various conditions including cotyledonary node explant, different plant growth regulators and concentration, medium components.

Effect of BA pretreatment and types of explant segment for shoot induction

Pretreatment with a high concentration of 200 ㎎/L BA onto explant before culture significantly promoted the number of shoots per explants compared with non-pretreated tissues (Fig. 3). Both of half-split cotyledon and cotyledonary node explants showed high frequency of shoots. All of explants produced shoot on the shoot induction with pretreatment or without pretreatment. The highest number of shoot per explant was shown in half-split pretreatment with 3.75 shoots per explant (Fig. 4). However, half-spit cotyledon and cotyledonary node with pretreatment or non-pretreatment did not make a significant difference.

In many reports, pretreatment of explants with plant growth regulators is the general technique to improve the shoot induction (Thomas, 2007; Tie *et al*., 2013). This performance is in line with the previous results. Tie *et al*. (2013) reported that pretreatment with 3 ㎎/L BA gave the best response in terms of shoot number and shoot length on cotyledonary node of cowpea.

Histological study on shoot formation

The histological observation provided evidence for direct formation of organogenesis structures at the cotyledonary node region in pea cv. 'Sparkle'. The results correlated with the cell formation and differentiation that occurred during shoot regeneration. In pea, cell division and proto-meristem formed near the surface of the sub-epidermal and epidermal cell layers of the cotyledonary node. Cell differentiation was initiated to develop early than 3 days (Fig. 5). Cell divisions leading to the formation of shoot primordial were occurred by 6-day. The apical region became wider due to the divisions of new cell layers. Anticlinal division of main vascular tissue

Fig. 3. Shoot regeneration by pretreatment with BA onto explants in half-split cotyledon (A, B) and cotyledonary node explant (C, D). Explants were pretreated with 200 mg/L BA for 1 min on the basal MS with B5 vitamin and 2 ㎎/L BA (A, C) or not (B, D) in 21 days.

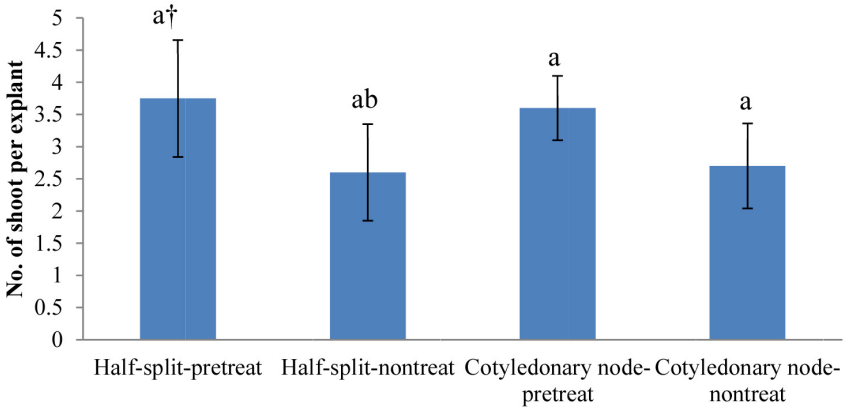

Fig. 4. Number of shoots per explants by different types of explants (half-split cotyledon or coteledonary node) and pretreatment onto explants. Explants were pretreated with 200 ㎎/L BA for 1 min. on shoot induction solution containing BA 2 ㎎/L. †Within each sampling date, the results followed by the same letters are not significantly different according to DMRT ($p < 0.05$).

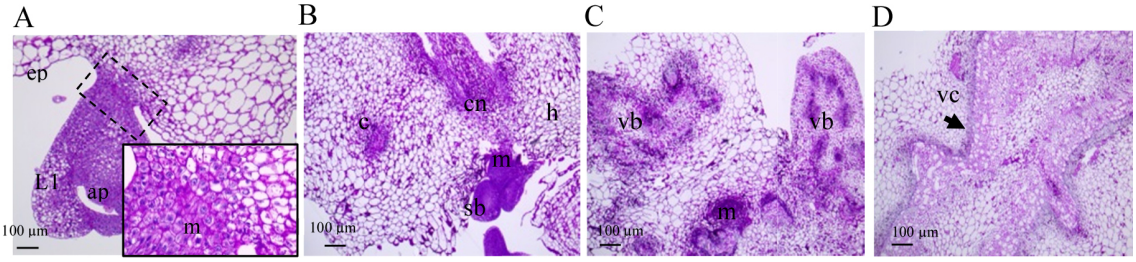

Fig. 5. Histological analysis of the shoot induction process in *Pisum sativum* cv. 'Sparkle'. The cotyledonary nodes were cultured for 0 to 21 days on the basal MS with B5 vitamin and 2 ㎎/L BA. A: 3-day, B: 6-day, C: 12-day, D: 18-day. Abbreviations; ap: apical meristem, cn: cotyledonary node, ep: epidermal cells, h: hypocotyl area, L1: leaf primodia, m: meristem cells, vb: vascular bundle, vc: vascular cambium.

Table 2. Thirteen ISSR primers and number of polymorphic bands amplified by each primers between regenerated plantlets and mother plants in pea cv. 'Sparkle'

Primers	(Sequence)[z]	TB[y]	PB[x]	PR[w]
UBC 811	$(GA)_8C$	2	0	0.0
UBC 827	$(AC)_8G$	3	1	33.3
UBC 835	$(AG)_8YC$	7	2	28.5
UBC 841	$(GA)_8YC$	3	1	33.3
UBC 856	$(ACA)_3(CAC)_2CYA$	4	0	0.0
UBC 861	$(ACC)_5$	5	0	0.0
UBC 862	$(AGC)_5$	4	0	0.0
UBC 864	$(ATG)_6$	6	0	0.0
UBC 866	$(CTC)_5$	2	0	0.0
UBC 880	$(GGAGA)_3$	3	0	0.0
UBC 889	$DBD(AC)_7$	3	0	0.0
UBC 891	$(TGT)_2(GTG)_2HVHTG$	3	0	0.0
UBC 893	$(NNN)_5$	3	0	0.0
Total bands		48	4	8.3

[z]B: C/G/T, D: A/G/T, R: A/G, Y: C/G, [y]Number of total bands, [x]Number of polymorphism bands, [w]Polymorphism rate.

and the main vascular and pro-vascular (new shoot) cambium was linked in 18 days.

The experiment showed that shoots were developed from epidermal cells, and surrounding cells differentiated similar as Klu (1996) indicated that shoot buds from cotyledonary node explant of winged bean were generated from sub epidermal parenchyma cells of the node. It revealed that the organogenesis often involves more than one cell that act in a coordinate manner identified by Brown and Thorp (1986). After new meristem formed a cell mass, then cell expansion and differentiation are required in control of plant hormones such as auxins and cytokinins (Reinhardt *et al*., 2003; Tran *et al*., 2016) and exogenous plant growth regulators enhance hormone signal.

Genetic stability of regenerants by ISSR markers

We evaluated the genetic stability among the clones of regenerants compared with their mother plant by ISSR molecular markers. Thirteen ISSR markers were used for genetic stability testing in all each regenerants. Among ten regenerants and their three mother plant, total of 48 alleles, were detected. Each ISSR primer generated a set of amplification products of size ranging from approximately 250 to 1,600 bp. The number of bands amplified in each ISSR primer ranged from 2 to 7. ISSR patterns of ten primers (UBC811, UBC856, UBC861, UBC862, UBC864, UBC866, UBC880, UBC889, UBC891 and UBC893) from regenerated plants were similar to the mother plant (100% monomorphic), whereas the rest primers found polymorphic bands with the total polymorphic rate of 8.3% (Table 2). Eight plates of the polymorphism bands (UBC827, UBC835 and UBC841) and the non-polymorphism bands (UBC811, UBC866, UBC880, UBC889 and UBC891) were shown in Fig. 6.

To maintain the genetic fidelity in regenerants, the clonal pattern of *in vitro*-regenerated plants is significant for confirmation of reliability of the protocol for mass propagation (Al-Qurainy *et al*., 2018). Our results were in accordance to the previous report on genetic stability of *in vitro* derived plantlets of *Platanus acerifolia* using ISSR markers (Huang *et al*., 2009). ISSR profiles of micropropagated plants were similar to the mother plants (Nayak *et al*., 2011; Mariappan *et al*., 2016). The occurrence of variations during *in vitro* propagation depends upon the source of explants and the pathway of regeneration (Goto *et al*. 1998). During *in vitro* culture some changes that cannot be investigated due to the different structure in the gene product do not always alter its

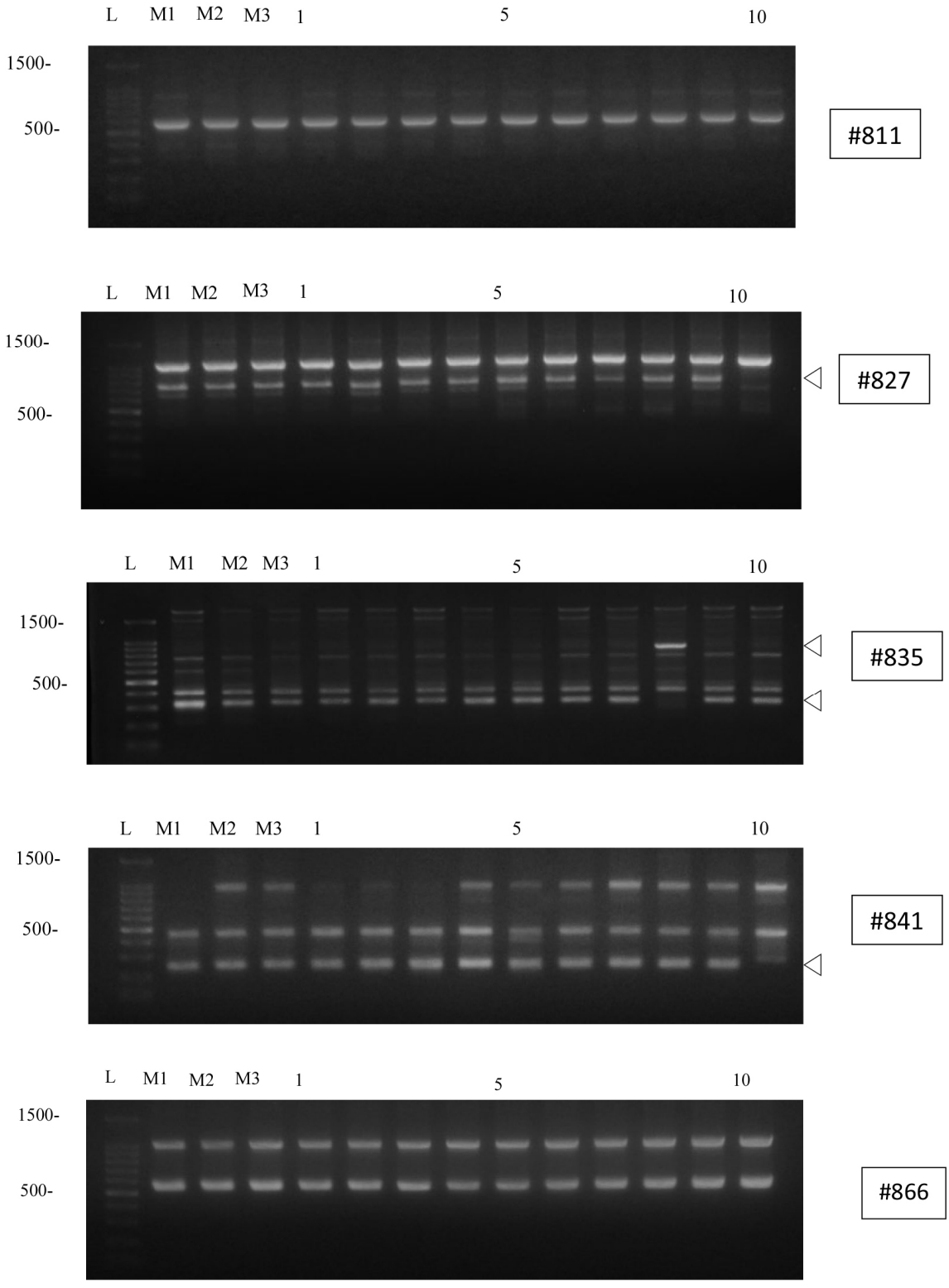

Fig. 6. ISSR finger-prints generated using 13 primers from 30 accessions of *Pisum sativum* cv. 'Sparkle'. Lane M: 100 bp DNA ladder marker, Lane M1-M3: mother plant, Lane1-10: regenerated plants. Arrows means positions of 4 polymorphic bands.

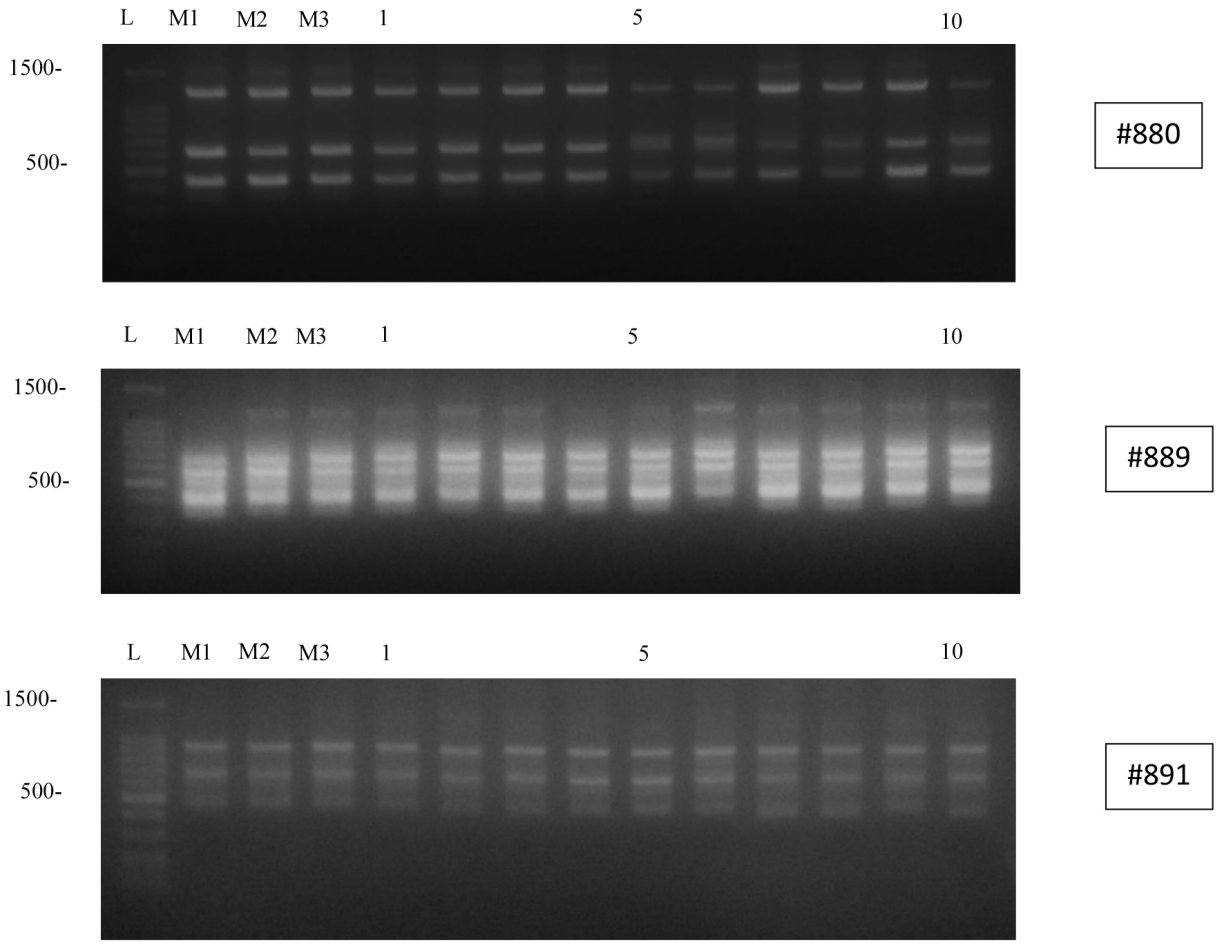

Fig. 6. Continued.

biological activity to an extent for phenotypic expression (Nayak *et al*., 2012). However, no marker analysis alone can completely guarantee the genetic fidelity of regenerated plants (Mallo'n *et al*. 2010).

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