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Detection of DNA methylation pattern in thidiazuron-induced blueberry callus using methylation-sensitive amplification polymorphism

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Abstract

During the normal developmental process, programmed gene expression is an essential phenomenon in all organisms. In eukaryotes, DNA methylation plays an important role in the regulation of gene expression. The extent of cytosine methylation polymorphism was evaluated in leaf tissues collected from the greenhouse grown plants and in *in vitro*-derived callus of three lowbush and one hybrid blueberry genotypes, using methylation-sensitive amplification polymorphism (MSAP) technique. Callus formation started from the leaf segments after 4 weeks of culture on a thidiazuron (TDZ) containing medium. Maximum callus formation (98 %) was observed in the hybrid blueberry at 1.0 mg dm^{-3} TDZ. Although noticeable changes in cytosine methylation pattern were detected within the MSAP profiles of both leaf and callus tissues, methylation events were more polymorphic in calli than in leaf tissues. The number of methylated CCGG sites varied significantly within the genotypes ranging from 75 to 100 in leaf tissues and from 215 to 258 in callus tissues. Differences in the methylation pattern were observed not only in a tissue-specific manner but also within the genotype in a treatment specific manner. These results demonstrated the unique effect of TDZ and the tissue culture process on DNA methylation during callus development.

Additional key words: gene expression, *in vitro* culture, *Vaccinium angustifolium, V. corymbosum*.

Introduction

Successful establishment of *in vitro* propagation systems enables rapid production of genetically similar plants. Although tissue culture propagated plants are intended to be identical, this is not always the case. Somaclonal variation (Larkin and Scowcroft 1981) occurs invariably during cell and tissue culture processes regardless to the regenerating system (Duncan 1997). Due to the occurrence of somaclonal variation in the micro-propagation system, clones are not true-to-type to the donor plants. Continuous subculturing and changes in the tissue culture microenvironment contribute to the additional stress to regenerating plant cells and induce a variety of genetic and epigenetic instabilities in the genome of the regenerants leading to the production of plant variants (Smýkal *et al.* 2007). In plants, epigenetic modification such as DNA methylation is associated with gene regulation, chromatin inactivation, genomic imprinting and cell differentiation (Park *et al.* 2009). DNA methylation induced changes have been hypothesized as the fundamental mechanism of tissue culture-induced mutations, which involves activation of transposable elements, chromosome breakage, and/or DNA sequence changes, and finally high frequency phenotypic variation (Kaeppler *et al.* 2000, Park *et al.* 2009). Across different taxa, methylation of the nucleotides is the most commonly found covalent modification of DNA (Fu *et al.* 2015). During post replicative DNA modification, specific DNA methylases generate several methylated bases (Wion and Casadeús 2006). Among these, 5-methylcytosine is predominant in higher plants and eukaryotes (Finnegan *et al.* 1998).

Methylation-sensitive amplification polymorphism (MSAP), a modification of the amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995), is a cost effective, rapid and easy to carry out process for non-model organisms and does not require information of the sequenced genome (Fulneček and Kovařík 2014). The technique is comparatively new and was used to

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Abbreviations: AFLP - amplified fragment length polymorphism, BM - basal medium; MSAP - methylation-sensitive amplification polymorphism, PPFD - photosynthetic photon flux density; TDZ - thidiazuron.
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determine DNA methylation events in dimorphic fungi (Reyna-López *et al.* 1997) and some plant species including rice (Xiong *et al.* 1999), banana (Peraza-Echeverria *et al.* 2001), Siberian ginseng (Chakrabarty *et al.* 2003) and in orchid *Doritaneopsis* (Park *et al.* 2009). MSAP technique is based on the sensitivity of the pair of isoschizomers *MspI* and *HpaII* instead of *MseI* used in the original AFLP protocol, to detect the methylation at the tetranucleotide recognition site CCGG (Peraza-Echeverria *et al.* 2001, Fulneček and Kovařík 2014). *MspI* and *HpaII* restrict differently the same recognition site depending on the methylation state of the external and internal cytosine residues. *MspI* cleaves methylated internal cytosine residues (C^mCGG) but not the external (^mCCGG), whereas the cleaving activity of *HpaII* is still under controversy. *HpaII* is most probably inactive for any methylation of CCGG site including hemimethylated external cytosine (^mCCGG, Tardy-Planechaud *et al.* 1997) and specific *HpaII* bands represent more likely fragments with internal C^mCGG site(s) which can be easily verified by combined digestion with both *HpaII* and *MspI* enzymes as suggested by Fulneček and Kovařík (2014).

Blueberry (*Vaccinium* spp. L., family: *Ericaceae*) is a widely accepted economically important health-promoting small fruit crop. In Newfoundland and Labrador, Canada, naturally growing wild lowbush blueberries are managed and harvested commercially to meet the increasing demand of high quality blueberries. Lowbush blueberry (*V. angustifolium* Ait.; 2n=4x=48) plants are hermaphroditic and predominantly cross-pollinated in nature (Vander Kloet 1978), and reproduces sexually by bee-pollination or asexually by underground rhizome system (Bell *et al.* 2009). Half-high blueberries are hybrids between highbush (*V. corymbosum*) and lowbush blueberries are outcrossing in nature (Harrison *et al.* 1993). Being genetically heterozygous in nature, blueberry plants grown from the seeds are not true-to-type to the donor plants. Although the species is successfully propagated by conventional methods, it is labour-intensive and time consuming. With the advent of plant tissue culture techniques, it is extensively employed to multiply plants rapidly which provides year around production. However, occurrence of somaclonal variation in micropropagated plants is a matter of concern as it affects clonal fidelity. Goyali *et al.* (2015) found that micropropagated lowbush blueberries contain a higher amount of polyphenols and flavonoids in fruits than softwood cutting counterparts, although number of flower clusters, berries, fruit mass per plants as well as diameter and mass of individual fruits were significantly lower in micropropagated plants. Variation originated in the lowbush blueberry clones might be due to the epigenetic changes occurred during tissue culture process, as molecular analysis of the micropropagated plants with simple sequence repeat (SSR) markers confirmed their genetic uniformity with the softwood cutting plants. The objective of this study was to estimate the altered methylation pattern at the tetranucleotide CCGG sites induced exclusively due to the tissue culture process at the stage of callus development with the application of thidiazuron (TDZ), having both the effects of auxin and cytokinin, using MSAP technique. To the extent of our knowledge, this is the first report on tissue culture induced DNA methylation in the genus *Vaccinium*.

Materials and methods

***In vitro* culture:** Actively growing young leaves were collected from wild lowbush blueberry (*Vaccinium angustifolium* Ait.) clones designated as CL1, CL2, CL3, and a hybrid (H1) between highbush blueberry (*Vaccinium corymbosum* L.*)* cvs. Chippewa and Patriot. The plants were maintained in plastic pots containing peat + *Perlite* (2:1, v/v) medium under the natural irradiance (photosynthetic photon flux density, PPFD of 90 µmol m^{-2} s^{-1}), a temperature of 20 ± 2 °C, and 85 % relative humidity in a greenhouse of St. John's Research and Development Centre, Agriculture and Agri-Food Canada, St. John's, Canada (Debnath 2009). Surface sterilized leaves (Debnath 2009) of each genotype were segmented into basal, middle and upper portion and inoculated abaxial surface touching the media in *Fisherbrand*™ Petri dishes with clear lids (*Fisher Scientific*, Fair Lawn, NJ, USA) containing 25 cm^3 of basal medium [BM, three-quarter micro salts and macro salts of Debnath and McRae (2001) supplemented with 25 g dm^{-3} sucrose, 1.25 g dm^{-3} *Gelrite*, and 3.5 g dm^{-3} *Sigma A 1296* agar (*Sigma Chemical Co.*, St. Louis, USA)]. The pH of the medium was adjusted to 5.0 before autoclaving at 121 °C for 20 min. BM was supplemented with four concentrations of TDZ, *i.e.*, 0 (control), 0.1, 0.5, and 1.0 mg dm^{-3}. The inoculated cultures were kept under dark for 2 weeks at 20 ± 2 °C, 60 - 70 % of relative humidity and then transferred to continuous PPFD of 30 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps under the same culture conditions. Calli were subcultured in the same media after every 4 weeks. Three explants per plate and three plates per treatment were used. The experiment was repeated three times.

DNA extraction: Genomic DNA was extracted from the leaf samples of greenhouse grown plants and from 12-week-old pale green clump of calli of all four genotypes using *DNeasy* plant mini kit (*Qiagen*, Hilden, Germany) as per the manufacturer instruction. The purity of the DNA was inferred from the absorbance ratio A_{260}/A_{280} and concentration from A_{260}. For MSAP analysis, the dilution of DNA used was 80 ng mm^{-3} with A_{260}/A_{280} of 1.8 - 2.1 (Debnath 2014).

MSAP assay was adopted from the AFLP protocol established by Vos *et al.* (1995) and modified by Reyna-López *et al.* (1997). *EcoRI* (*Thermo Scientific,* Burlington, ON, Canada) and the methylation-sensitive restriction enzymes (isoschizomers), *MspI* and *HpaII* (*Thermo Scientific*) were used. To detect MSAP, digestion reactions were set up in two steps. In the first step, 400 ng of the genomic DNA was digested with 2 U of *EcoRI* in a final volume of 50 mm^3 containing 10× *FastDigest* buffer (*Thermo Scientific*) at 37 °C for 1.5 h and the reaction was stopped by incubating at 65 °C for 15 min. In the second step, two digestion reactions were carried out simultaneously. In the first reaction, 25 mm^3 of *EcoRI* digested DNA was restricted with 2 U of *MspI* enzyme in a final volume of 50 mm^3 containing 10× *FastDigest* buffer. The second reaction was carried out in the same way except *HpaII* enzyme instead of *MspI* enzyme. Both reaction mixtures were incubated at 37 °C for 3 h followed by stopping the reaction at 65 °C for 15 min. In another reaction, 400 ng of the genomic DNA was digested with 2 U of each of *EcoRI*, *MspI*, and *HpaII* enzymes in a final volume of 50 mm^3 containing 10× *FastDigest* buffer for 3 h at 37 °C. The reaction was stopped by incubating the mixture at 65 °C for 15 min.

All digested fragments were ligated to the adapters by adding 50 mm^3 of ligation mixture containing 1× T4 DNA ligase buffer (*Thermo Scientific*), 10 pmol *EcoRI* adapter, 100 pmol *MspI-HpaII* adapter (*Thermo Scientific*), 50 % (m/v) polyethylene glycol solution and 5 U of *T4* DNA ligase (*Thermo Scientific*) and incubated at 23 °C for 5 h. The reactions were stopped at 65 °C for 10 min.

Pre-selective amplification was conducted by using 4 mm^3 of the ligated product with *EcoRI and MspI-HpaII* primers in a volume of 50 mm^3 containing 1× Taq buffer (*Qiagen*), 10 mM dNTPs (*Amresco LLC*, Solon, OH, USA), 10 mg dm^{-3} of each primer and 5 U of Taq polymerase (*Qiagen*). The PCR was performed with the following profile: 65 °C for 2 min, 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 2 min with a final extension at 72 °C for 10 min. After checking for the smear of fragments (100 - 1000 bp in lengths) in 1.8 % agarose gel electrophoresis, the amplification product was diluted to 15 times in 1× Tris-borate-EDTA (TBE) buffer and stored at -20 °C until used for selective amplification.

Selective amplification reactions were conducted in volumes of 25 mm^3 containing 2 mm^3 of the preamplified DNA, 10 mg dm^{-3} of *EcoRI* primer, and 10 mg dm^{-3} of *MspI-HpaII* primer along with the same other components as used in pre-selective amplification step. Selective amplification involved 94 °C for 5 min, 13 cycles of 94 °C for 30 s, 65 °C for 1 min, 72 °C for 2 min followed by 23 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 2 min with a final extension at 72 °C for 10 min.

MSAP electrophoresis and autoradiography: The selective PCR products were mixed with equal amount of formamide dye [98 % (v/v) formamide, 10 mM EDTA, 0.01 % (m/v) bromophenol blue and 0.01 % (m/v) xylene cyanol)], denatured at 95 °C for 4 min and immediately cooled on ice. Aliquots (6 mm^3) of each reaction were separated by electrophoresis on 5 % denaturing polyacrylamide sequencing gels (5 % acrylamide 19:1, 7 M urea) in 1× TBE buffer. The gels were pre run at 100 V for 30 min, 6 mm^3 of each of the PCR product and formamide dye mix were loaded, and the gels were run at 115 V for 5 h in *Owl* gel separation system (*Thermo Scientific*). Silver staining was done following Bassam *et al.* (1991). The gel was fixed in the fixer solution (7.5 % acetic acid) for 30 min, washed twice with deionized double distilled water and incubated in the silver solution (0.1 % $AgNO_3$) for 30 min. The silver impregnated gel was washed twice with ultrapure water collected from *Barnstead Mega-Pure D2* (*Thermo Scientific*). Image development was carried out in the developer solution (30 g dm^{-3} sodium carbonate, 0.056 % formaldehyde, 40 g dm^{-3} sodium thiosulfate) for 1 - 2 min. To stop the image development, developer stop solution (7.5 % acetic acid) was added immediately and incubated for 3 min. All steps were performed with slow agitation on *Rocker 25* shaker (*Labnet International,* Edison, NJ, USA). The gel was then rinsed with deionized double distilled water and dried at room temperature. Band separation pattern was visualized using the *InGenius 3* gel documentation system (*Syngene*, Frederick, MD, USA).

Data analysis: Callus formation experiments were conducted following completely randomized design (CRD) and the data on callus formation were subjected to analysis of variance (*ANOVA*) employing the general linear model. The significance of the TDZ treatments was assessed by Duncan Multiple Range Test (DMRT) (Duncan 1955) using *STATISTICA* data analysis software version 7 (*Statsoft Wipro*, East Brunswick, NJ, USA) at a critical difference of $P \leq 0.05$.

In the MSAP analysis, DNA methylation event was detected on the basis of the presence or absence of the bands in the autoradiograph. Methylated tetranucleotide sites (CCGG) were detected when *MspI* cleaved only internal methylated cytosine (C^mCGG) residues. Any extra pure *HpaII* bands were not detected for the digestion of hemimethylated external cytosine (^mCCGG) and the fact was supported by the third lane of the samples digested with *MspI + HpaII* enzyme combinations, which showed almost same profile as *MspI* (Fulneček and Kovařík 2014). Polymorphic bands for DNA methylation events were recorded in all reactions with *EcoRI + MspI/HpaII/MspI+HpaII* following Chakrabarty *et al.* (2003).

Results and discussion

Leaf explants of all genotypes, when cultured *in vitro* on medium with TDZ, formed callus (Fig. 1). Pale green callus started emerging at the leaf margin after 4 weeks of culture on BM containing 0.1, 0.5, and 1.0 mg dm^{-3} TDZ. Cultures on BM without TDZ did not form any callus and were excluded from data analysis. After 12 weeks of culture, the highest percentage of callus formation was observed in H1 (98 %) at 1.0 mg dm^{-3} TDZ. The callus formation increased with the increase of TDZ concentration in the culture medium for H1 but not for the clones where the highest percentage of callus formation was observed at 0.5 mg dm^{-3} TDZ (Fig. 2).

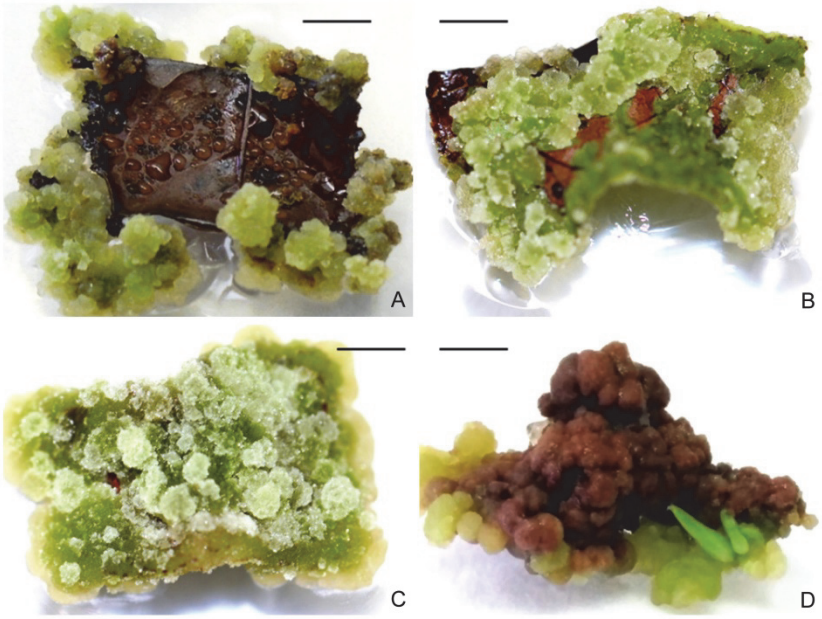

Fig. 1. Callus and shoot formation on hybrid blueberry H1 (Chippewa × Partriot) leaf explants. Callus forming after 4 (*A*), 8 (*B*), and 12 weeks of culture (*C*). Formation of shoots from the callus clump after 16 weeks of culture (*D*). *Bar* = 2 cm.

Although in all four genotypes maximum numbers of methylated CCGG sites were recorded in the callus with 1.0 mg dm^{-3} TDZ, the number of methylated sites detected varied among the genotypes. For example, in hybrid H1, the total number of methylated sites were 239 (Table 1), among which the highest number of CCGG methylation detected were 93 in the callus formed on BM with 1.0 mg dm^{-3} TDZ, followed by 81 at 0.1 mg dm^{-3} TDZ and 65 at 0.5 mg dm^{-3} TDZ (Table 2). In CL1, CL2, and CL3, total number of methylated tetranucleotide sites were recorded 215, 237, and 258, respectively (Table 1). In the callus tissues of these three clones, the maximum methylated sites were 81, 80, and 94, respectively, at 1.0 mg dm^{-3} TDZ followed by 77, 79, and 86, respectively, at 0.1 mg dm^{-3} TDZ. At 0.5 mg dm^{-3} TDZ, these values were 57, 78, and 78, respectively (Table 2). From these observations it is clear that all genotypes followed the same trend in relation to DNA methylation at different TDZ concentrations (Table 2). It is reported that in dedifferentiation stage (callus formation), explants change dynamically and cytokinins are apparently effective for genome modification during that stage. In support of that fact, LoSchiavo *et al.* (1989) observed that upon applying exogenous cytokinin, a stable methylation pattern with 16 % methylated cytosine was expressed in cell suspension culture of carrot. The same trend was reported by Arnholdt-Schmitt *et al.* (1995) where freshly inoculated tissues of carrot showed a higher rate of methylation than established cell culture with the application of kinetin. In our study, the reason behind the hypermethylation status observed in the *in vitro* formed callus might be TDZ present in the media.

Although all living cells of the plants are theoretically totipotent in nature to acquire dedifferentiation, redifferentiation, and competency; application of plant growth regulators is essential (Murthy *et al.* 1998). During explant culture *in vitro*, TDZ mimics the effect of auxin and cytokinin and helps in the differentiation process alone or in combination with other phytohormones (Murthy *et al.* 1998, Ghosh *et al.* 2014). With the application of different concentrations of TDZ in the medium, callus formation from the leaf segments up to 78 % was reported in lowbush blueberry by Debnath (2009). Callus formation due to the application of TDZ was observed earlier in woody species such as grape. In Kyoho grapes (*Vitis vinifera* × *Vitis labruscana* cv. Kyoho), with the linear increment of TDZ concentration, maximum callus growth was obtained after 12 d of culture in medium supplemented with 100 mg dm^{-3} TDZ (Lin *et al.* 1989).

Table 1. Total number of methylated CCGG sites and the number of methylation polymorphism for each primer detected by methylation-sensitive amplification polymorphism in tissue culture derived calli of four blueberry genotypes. Each experiment was repeated twice with three independently induced calli for each treatment and the same bands were detected in each case.

Oligonucleotide combination	Total number of methylated CCGG sites				Methylation polymorphism			
	CL1	CL2	CL3	H1	CL1	CL2	CL3	H1
E-TT/MH-AAC	9	16	20	17	5	2	2	6
E-TT/MH-ATG	8	9	10	8	2	2	2	2
E-TT/MH-AAG	6	3	14	8	3	0	4	4
E-TT/MH-ACA	46	35	24	26	8	3	3	4
E-TG/MH-AAC	65	61	67	64	7	4	6	8
E-TG/MH-ATG	16	24	36	31	3	4	6	7
E-TG/MH-AAG	38	46	50	44	5	7	7	8
E-TG/MH-ACA	27	43	37	41	7	4	2	7
Total	215	237	258	239	40	26	32	46

Leaves from greenhouse grown plants and *in vitro*-derived callus of four blueberry genotypes were used for MSAP analysis. We used eight primer combinations (Table 1 Suppl.) to detect the cytosine methylation at the tetranucleotide (CCGG) recognition sites. The adapter and primers were designed following Xiong *et al.* (1999). Leaf and callus DNA digested with *MspI* and *HpaII* enzymes can produce three fragment classes: *1*) cleaving non-methylated CCGG tetranucleotide sites, both and *HpaII* produce identical fragments, *2*) *MspI* specific fragments result from digestion of fully or hemi-methylated internal cytosine (C^mCGG) in the recognition site; and *3*) *HpaII* specific fragments assumed to represent hemimethylated external cytosines (^mCCGG) (Reyna-López *et al.* 1997) are more likely fragments with CCGG site at the end and one or more internal C^mCGG sites which can be verified by adding one or more lane after simultaneous *MspI* + *HpaII* digestions as recommended by Fulneček and Kovařík (2014). The reproducibility of the methylation patterns was confirmed by repeating the experiments and all the bands were repeatedly detected in each case (Fig. 3).

The primer combinations produced a total of 75 fragments in H1 leaves which were methylated at CCGG sites, out of which 10 sites were polymorphic. In CL1, CL2, and CL3, total number of polymorphic sites were 10, 9, and 8, respectively (Table 3). Calli from all genotypes and concentrations of TDZ were also analyzed for altered methylation pattern where highest methylation was recorded in CL3 (258 fragments) and the lowest in CL1 (215 fragments produced) for the eight specific primer combinations. CL2 and H1 were differentially amplified with 237 and 239 bands, respectively (Table 1). The number of polymorphic events increased significantly in *in vitro* grown callus compared to leaves. These values were 40, 26, 32, and 46, respectively for CL1, CL2, CL3, and H1 calli (Table 1). The increased polymorphism of callus tissues compared to those in leaves might be due to the stress induced by tissue culture process and the influence of TDZ on methylation. Banding pattern detected in leaf tissues (Table 3) and in callus cultures was genotype dependent (Table 1). Our results have demonstrated the effect of TDZ concentrations on number of methylation events in each genotype (Table 2). All genotypes responded in the same manner, in terms of total number of methylated tetranucleotide sites in the callus, treated with 0.1, 0.5, and 1.0 mg dm^{-3} TDZ. The number of methylated CCGG sites varied from 80 (CL2) to 94 (CL3) in callus cultures at 1.0 mg dm^{-3} TDZ. However, the presence of polymorphic sites among the methylated CCGG sites was highest (16.7 to 27.7 %) at 0.5 mg dm^{-3} TDZ for all four genotypes (Table 2).

The present results are in agreement with those found in micropropagated banana (Peraza-Echeverria *et al.* 2001) and in seedlings and flag leaves of rice (Xiong *et al.* 1999), where the methylation pattern was found to be tissue specific. This is most likely due to the reason that DNA methylation is a tissue specific developmentally regulated phenomenon (Chakrabarty *et al.* 2003). Evidences are available on spontaneous DNA methylation variants that arise in tissue culture system and do not follow Mendelian inheritance (Niederhuth and Schmitz 2014). As calli go through the dedifferentiation and redifferentiation process during regeneration, the level of methylation changes drastically (Huang *et al.* 2012). The reason behind changed methylation pattern during the developmental process is for the failure of maintaining the methylation status during DNA replication due to *de novo* methylation and passive demethylation (Hsieh 1999). This might have caused higher frequency of methylation polymorphism in *in vitro* grown callus compared to greenhouse-grown leaves. Plants predominantly do not undergo the cycle of remethylation following demethylation in the tissue culture system and thus produce variants. For example, in *Arabidopsis thaliana*, decreased methylation gave rise to numerous morphological and phenotypic abnormalities including decreased apical dominance, reduced plant size, modified leaf size and shape, diminished fertility, and altered flowering time as the plants were unable to restore the previous methylation pattern after the passage through meiotic event (Finnegan *et al.* 1996).

In the current study, calli for all genotypes were

maintained under same *in vitro* condition except different TDZ treatments. Altered methylation pattern in the calli might be due to the effect of TDZ concentration in the culture. The role of phytohormones has been proposed to play the central role to control the signal transduction cascade leading to the reprogramming of the gene expression (Von Arnold *et al.* 2002). With the three different concentrations of TDZ, the variable pattern of cytosine methylation originated within the same genotype, has similarities with the cases of tissue cultured carrot plants, where different treatments of phytohormones altered methylation pattern (LoSchiavo *et al.* 1989, Arnholdt-Schmitt *et al.* 1995).

DNA methylation is an epigenetic phenomenon associated with somaclonal variation, which has potential use in the conventional plant breeding program (Karp 1995). However, it has negative implications for clonal propagation and affects the production of true-to-type plants to the donor plants. So, the maintenance of clonal fidelity is of prime interest in tissue culture system

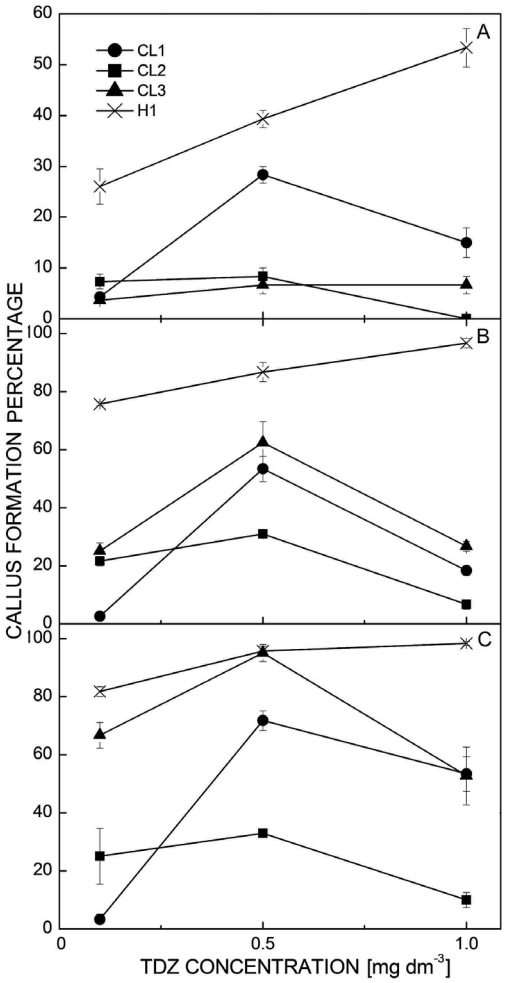

Fig. 2. Percentage of callus formation of lowbush blueberry clones CL1, CL2, CL3 and hybrid H1 after 4 (*A*), 8 (*B*), and 12 weeks of culture (*C*) on BM medium supplemented with 0.1, 0.5, and 1.0 mg dm^{-3} TDZ (Means ± SEs, $n = 3$).

Table 2. Effect of thidiazuron (TDZ) concentrations on the total number of methylated CCGG sites, number of methylation polymorphism, and percentage of methylation polymorphism for CCGG sites detected within four blueberry genotypes. Each experiment was repeated twice with three independently induced calli for each treatment and same bands were detected in each case.

Genotype	TDZ [mg dm^{-3}]	Total number of methylated CCGG sites	Number of methylation polymorphism	Percentage of methylation polymorphism
CL1	0.1	77	9	11.7
	0.5	57	13	22.8
	1.0	81	18	22.2
CL2	0.1	79	7	8.9
	0.5	78	15	19.2
	1.0	80	4	5.0
CL3	0.1	86	8	9.3
	0.5	78	13	16.7
	1.0	94	11	11.7
H1	0.1	81	14	17.3
	0.5	65	18	27.7
	1.0	93	14	15.1

Table 3. Total number of methylated CCGG sites and the number of methylation polymorphism for each primer detected by methylation-sensitive amplification polymorphism in the greenhouse-grown leaves of four blueberry genotypes. Each experiment was repeated twice and the same bands were detected in each case.

Oligonucleotide combination	Total number of methylated CCGG sites				Methylation polymorphism			
	CL1	CL2	CL3	H1	CL1	CL2	CL3	H1
E-TT/MH-AAC	14	10	10	12	0	0	1	1
E-TT/MH-ATG	15	14	11	17	1	1	2	1
E-TT/MH-AAG	12	2	10	6	3	1	1	1
E-TT/MH-ACA	9	12	12	2	1	0	1	0
E-TG/MH-AAC	15	13	14	10	0	2	1	2
E-TG/MH-ATG	6	7	10	7	2	1	0	2
E-TG/MH-AAG	15	15	14	9	1	1	2	2
E-TG/MH-ACA	14	15	16	12	2	3	0	1
Total	100	88	97	75	10	9	8	10

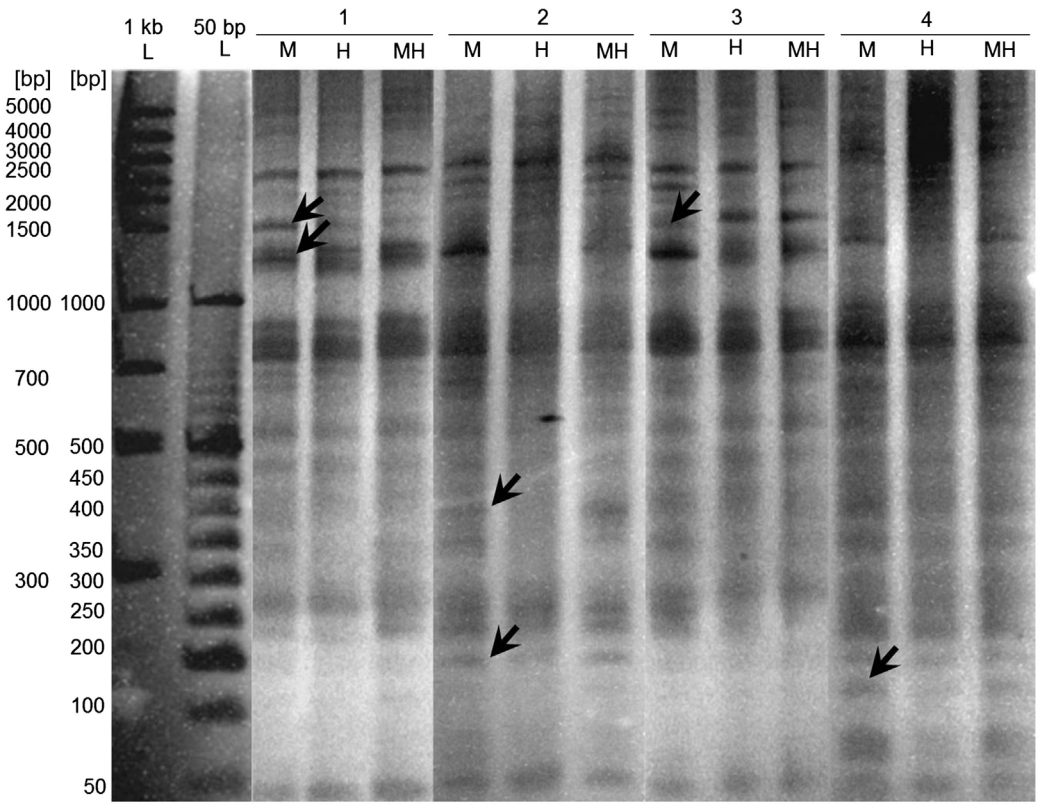

Fig. 3. Methylation pattern detected in lowbush blueberry clones CL1 and CL2. Pattern detected in *in vitro* grown callus of CL1 (1), CL2 (3) and greenhouse-grown leaves of CL1 (2) and CL2 (4) using the primer combination E-TG/MH-ACA. M, H and MH refers to the digestion with *EcoRI* + *MspI*, *EcoRI* + *HpaII* and *EcoRI* + *MspI* + *HpaII* enzymes, respectively. 1 kb L - MidRanger 1 kb DNA ladder (*Norgen*, Biotek Corp., Thorld, ON, Canada), 50 bp L - O'RangeRuler 50 bp DNA ladder (*Thermo Scientific*, Burlington, ON, Canada). *Arrows* show differentially methylated CCGG sites occurred in the enzyme digested DNA sequences of CL1 callus treated with 0.5 mg dm^{-3} TDZ (1), CL1 leaves (2), CL2 callus treated with 0.5 mg dm^{-3} TDZ (3), and CL2 leaves (4).

(Karp 1995). For example, production of 'an array of agronomically inferior genotypes' of spring wheat (*Triticum aestivum* cv. HY320) through tissue culture has been reported by Qureshi *et al.* (1992). Till date, the molecular basis of somaclonal variation is not completely understood. The most accepted hypothesis is that during *in vitro* culture, breakdown of normal cellular process results in genetic and epigenetic instabilities and altered gene expression that produces plant variants (Kaeppler *et al*. 2000). This may be due to the exposure to the stressful conditions such as wounding, pathogen attack, or the application of growth regulators in tissue culture media. Callus formation is a huge commitment for a fully grown plant system as during this phase, plants give up their established developmental program and switch to a new one (Ikeuchi *et al.* 2013). In tissue culture system, plant cells undergo dedifferentiation and redifferentiation stimulated with the introduction of a plant growth regulator in the culture medium. During this phase, formation of callus is induced by the termination of current gene expression program and is turned on the callogenic gene expression process. As a result, DNA methylation is induced in the callus formation and differentiation stage (Huang *et al.* 2012). Several authors demonstrated the changes of the methylation pattern in tissue culture plants (Park *et al.* 2009). However, manipulation in the tissue culture conditions during *in vitro* culture induces altered methylation status. LoSchiavo *et al.* (1989) showed in carrot cultures that global methylation patterns vary with the changed hormone concentration in the media. In this study, it was noticed that the level of methylation was developmentally regulated and changed with the induction of somatic embryogenesis. Arnholdt-Schmitt *et al.* (1995) observed that the DNA methylation varied in different developmental stages of tissue culture regenerated carrot plants. Our study provides the evidence that tissue culture process during callus formation was solely responsible for the induction of the hypermethylation pattern as the leaves from conventionally propagated plants showed lower DNA methylation compared to the *in vitro*-derived callus. To the best of our knowledge, this is the first report of DNA methylation at callus stage in blueberry or any other woody species cultured *in vitro*. Further studies are required at the plantlet stage to identify whether DNA methylation is changing from callus to the plantlet formation. Moreover, the characterization of the gene expression during callus formation and maturation, and

during different stages of plantlet formation will lead to the identification of several developmental regulatory genes that will further help in better understanding the methylation induction process in a plant tissue culture system.

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