

Genetic instability in calamondin (*Citrus madurensis* Lour.) plants derived from somatic embryogenesis induced by diphenylurea derivatives

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Abstract Somatic embryos were regenerated in vitro from calamondin style–stigma explants cultured in the presence of N^6 -benzylaminopurine (BAP) cytokinin and three synthetic phenylurea derivatives, *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (4-CPPU), *N*-phenyl-*N'*-benzothiazol-6-ylurea (PBU) and *N,N'*-bis-(2,3-methilendioxyphenyl)urea (2,3-MDPU). The phenylurea derivative compounds tested at micromolar level (12 μ M) were able to induce a percentage of responsive explants significantly higher from that obtained with BAP and hormone-free (HF) conditions. In order to verify the genetic stability of the regenerants, 27 plants coming from different embryogenic events were randomly selected from each different culture condition and evaluated for somaclonal variations using inter-simple sequence repeat and random amplified polymorphic DNA analyses. We observed that 2,3-MDPU and PBU gave 3.7% of somaclonal mutants, whereas 4-CPPU gave 7.4% of mutants. No somaclonal variability was observed when plantlets were regenerated in BAP or HF medium. Although diphenylurea derivatives show a higher embryogenic potential as compared to BAP, they induce higher levels of somaclonal variability. This finding should be taken in consideration when new protocols for clonal propagation are being developed.

Keywords Genetic polymorphisms · Molecular markers · Mutations · Plant regeneration · Somaclonal variability

Abbreviations

BAP	N^6 -benzylaminopurine
4-CPPU	<i>N</i> -(2-chloro-4-pyridyl)- <i>N</i> -phenylurea
HF	Hormone-free
ISSR	Inter-simple sequence repeats
2,3-MDPU	<i>N,N'</i> -bis-(2,3-methilendioxyphenyl)urea
NO	Nitric oxide
PBU	<i>N</i> -phenyl- <i>N'</i> -benzothiazol-6-ylurea
RAPD	Random amplified polymorphic DNA
ROS	Reactive oxygen species
TDZ	Thidiazuron

Introduction

The calamondin is a common ornamental tree frequently used in the landscape and for homeowner uses (Ferguson and Castle 1998) and it is also valued as a rootstock for the oval kumquat for pot culture (Mabberley 1987). It has also several different uses, as food (juices, sauces and marmalades), medicinal (antiphlogistic, for cough remedy and like laxative) and industrial product (as bleaching, deodorant and for hair cures).

Somatic embryogenesis is an in vitro-applied method that offers opportunities for clonal plant propagation and for regeneration of plants from genetic transformation and somatic hybridisation experiments. A wide range of organs and tissues are in vitro-cultivated to produce plants via somatic embryogenesis having some specific required characteristics, such as virus-free, vigorous, normally

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developed and genetically true-to-type (Cassells 2000; Cassells et al. 2000). In some cases, plantlets derived from in vitro culture and their progenies might to develop altered characteristics and reveal a wide array of culture-induced genetic variants (Philips et al. 1994; Lee et al. 1996; Chowdari et al. 1998; Yang et al. 1999) these modifications are called somaclonal variations (Larkin and Scowcroft 1981). Somaclonal variations may have their bases in epigenetic changes and be unstable (Nelson 1977; Schaeffer 1981) other somaclonal variations may derive from karyotype changes, chromosomal rearrangements and gene mutations (Muler et al. 1990; Kuksova et al. 1997; Palombi and Damiano 2002). These genetic changes can be inherited and occur at higher frequency than occurs spontaneously in seeds or grafted plants (Preil 1986). Therefore, somaclonal variations may constitute a source of new genetic variability that is an essential component of any breeding program designed to improve the characteristics of plants. On the contrary, they may represent a very serious problem for the preservation of the regenerated plant genetic integrity, where the most important concern is only the clonal propagation of the plants. Thus, several assays to identify genetic stability during in vitro culture procedures have been developed. Karyotypical, structural and flow cytometric analysis have been widely used for identification of chromosomal variations (Nkongolo and Klimaszewska 1995; O'Brien et al. 1996). DNA-based techniques, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) and microsatellites have been used for molecular characterization of tissue culture-derived plants (Jain and De Klerk 1998; Jain 2001; Martins et al. 2004).

In many studies, RAPD analysis has proved to be a straightforward method to identify genetic variations generated by in vitro culture techniques (Hashmi et al. 1997; Goto et al. 1998; Soniya et al. 2001). RAPD primers randomly scan the whole genome detecting DNA mutations (Milbourne et al. 1997). This analysis requires only a small amount of DNA and permits to analyse quickly and economically many samples. ISSR technique has showed the same advantages in several characterization studies (Fang et al. 1997; Fang and Roose 1997; Scarano et al. 2002; Lambardi et al. 2004). It detects genetic mutations at hypervariable sites, such as DNA repetitive regions, using highly specific 18-bp long primers. For this reason, this technique guarantees higher reliability and repeatability than RAPD technique (Bornet and Branchard 2001).

At present, it is known that the occurrence of somaclonal variation is influenced by the regeneration conditions (Karp 1991). In most culture systems, somatic embryogenesis is induced by modulation of the auxin to cytokinin ratio and absolute growth regulator concentrations (Skoog

and Miller 1957). While the role of auxins in the induction of somatic embryogenesis has been well described (Michalczuk et al. 1992), on the contrary the role of cytokinins in embryo induction and development appears extremely complex (Victor et al. 1999).

Cytokinin activity is a property of two classes of compounds: the N⁶-substituted adenine derivatives (Shaw 1994), and the synthetic phenylurea derivatives (Shudo 1994). The identification of diphenylurea (DPU) (Shantz and Steward 1955) was followed by the synthesis of a class of new analogous compounds, such as *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thiadiazuron, TDZ) and *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (4-CPPU), with even higher cytokinin activity than that of adenine type derivatives (Mok et al. 1982, 1987). Diphenylurea derivatives showed a positive role also in inducing somatic embryogenesis: 4-CPPU was used to induce somatic embryogenesis in floral explants of *Citrus* (Fiore et al. 2002) and thiadiazuron induced somatic embryogenesis in caryopses of rice, inducing shoot regeneration better than that obtained with *N*⁶-benzylaminopurine (BAP) treatment (Gairi and Rashid 2004). Recently, Ricci et al. (2001a, b) demonstrated that some diphenylurea derivatives showed biological activity in some biological assays. *N*-phenyl-*N'*-benzothiazol-6-ylurea (PBU) acted positively in inducing morphogenesis in tomato cotyledons and the *N,N'*-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) induced adventitious rooting in apple micro-cuttings.

It is already known that growth regulator composition of the culture medium can influence frequency of morphological and physiological alterations in cultured cells (Ziv 1991). Several growth regulators, such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA) and BAP, have been most frequently considered to be responsible for genetic variability (Chawla 2002; Gesteria et al. 2002; Rakoczy-Trojanowska 2002), but evidences about the possible effects of synthetic phenylurea derivatives on the genetic stability of regenerated plants are still lacking.

In this work, we evaluated the genetic stability of calamondin (*Citrus madurensis* Lour.) plants derived from somatic embryos regenerated in presence of phenylurea derivative compounds (4-CPPU, PBU and 2,3-MDPU). For this purpose, we used two different DNA-based techniques, ISSR and RAPD.

Materials and methods

Plant material

Calamondin flowers were collected before opening, during the period of full bloom, from plants growing in the field

(collection of the Institute of Plants Genetic, CNR). They were surface-sterilised by immersion for 5 min in 70% ethanol (v/v), 20 min in 2% (w/v) sodium hypochlorite, followed by three 5 min rinses in sterile distilled water.

Stigmas and styles were excised with a scalpel and plated vertically as unique explant into the media with the cut surface in contact with the medium.

Media and culture conditions

Somatic embryos and plantlets were obtained as previously described (Fiore et al. 2002) with minor modifications. Explants were cultured on MS solidified medium (Murashige and Skoog 1962) (8 g/l agar, B&V, Italy) with 500 mg/l malt extract (Sigma M-0383) and 146 mM sucrose as carbon source, under three different hormonal conditions: 4-CPPU (Sigma C-2791), PBU and 2,3-MDPU. As control, BAP (Sigma B-4308) and hormone-free supplemented media were used. For all the cultural conditions the hormone concentration was 12 μ M. PBU and 2,3-MDPU, synthesised as previously reported (Ricci et al. 2001a, b) were of analytical grade.

DNA extraction

DNA was extracted from young leaves of plants growing in the field (for the maternal plant) or in vitro (for the regenerated plantlets). Twenty-seven somaclones coming from different embryogenic events were randomly selected for each different culture condition for the analyses of genetic stability. R0 plants were considered coming from different embryogenic events when embryos were regenerated from different explants or from clearly defined, distinct region of the same explant. All the samples were frozen in liquid nitrogen and stored at -80°C . They were ground in a mortar with liquid nitrogen and genomic DNA was extracted using the procedure described by Doyle and Doyle (1987). DNA was quantified by measuring OD₂₆₀ as described by Sambrook et al. (1989).

ISSR analysis

A total of ten primers—i.e., (AC)₈YG, (AG)₈YC, (AC)₈YA, (AC)₈YT, (AG)₈YT, (GT)₈YG, (TCC)₅RY, (GA)₈YC, (CA)₈RG and (GA)₈YG reported by Fang and Roose (1997)—were used to amplify the DNA. The primers were purchased from Life Technologies, Gaithersburg, MD.

Each 25- μ L amplification reaction consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 800 μ M dNTP, 0.5 μ M of each primer, 1 U of Platinum *Taq* polymerase (Life Technologies) and 30 ng of template DNA. The amplification was performed in a MJ Research

thermocycler (Genenco) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 4 min at 94°C, followed by 36 cycles at 94°C for 30 s (denaturation), 47–52°C for 45 s (annealing) and 72°C for 120 s (extension), followed by a final extension step at 72°C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1 \times TBE (45 mM Tris-borate, 1 mM EDTA) and 0.5 μ g/ml aqueous solution of ethidium bromide. About 25 μ L of reaction products (with an adequate amount of loading buffer) were loaded and the gel was run for 4 h at 100 V. The gel was then visualised under UV light.

RAPD analysis

Six 10-mer primers—i.e., OPH04, OPAT14, OPH15, OPM04, OPO14 and OPN14 reported by Coletta Filho et al. (1998)—were used for the RAPD analysis. The primers were purchased from Life Technologies, Gaithersburg, MD.

DNA amplification reactions were performed in a volume of 25 μ L with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 800 μ M dNTP, 0.4 μ M of each primer, 1 U of Platinum *Taq* polymerase (Life Technologies) and 30 ng of template DNA. The amplification was performed in a MJ Research thermocycler (Genenco) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 90 s at 94°C, followed by 40 cycles at 94°C for 1 min (denaturation), 35°C for 2 min (annealing) and 72°C for 2 min (extension), followed by a final extension step at 72°C for 10 min. PCR-amplified DNA fragments were visualised as described above.

Data analysis

A total of ten replicates (petri dishes) and five organs per replicate were prepared. Each treatment comprised 50 explants. Percentages of responsive explants and number of embryos per explant were counted after 4 months of incubation. Embryogenic response of explants were expressed as percentages on a petri dish basis. Effects of treatment were tested by Analysis of Variance ($P \leq 0.01$).

For the molecular analysis, only those bands showing consistent amplification in the range of 200 bp to 2.6 kb were considered; smeared and weak bands were excluded. Polymorphic ISSR and RAPD markers were scored for the presence (1) or absence (0) of bands for all the somaclones analyzed. All the PCR-amplified samples coming from plantlets regenerated in the same culture conditions were run in the same gel compared with the PCR-product of the mother plant. To confirm the obtained polymorphisms, the analysis was repeated performing separate PCRs and the DNA of polymorphic plantlets were re-extracted and tested again.

The analyses of genetic diversity were conducted using the software POPGENE version 1.31 (Yeh et al. 1999). Dice's (1945) coefficient of similarity (D_{ij}) was determined between each pair of somaclones. Dice's coefficient has been recommended for the evaluation of genetic similarities when using RAPD markers (Lambooy 1994). The genetic distance (GD) between two samples was calculated as: $GD = 1 - D_{ij}$. Genetic diversity (H) of Nei (1973) was used to summarise the diversity induced by the different hormonal conditions. Standard deviations were indicated where it was necessary. The percentage of polymorphism (P) was given as number of polymorphic loci/number of total loci, regardless of allele frequencies. Additional statistics were computed to estimate the efficiency of ISSR and RAPD methods in detecting polymorphisms.

Results

Explants produced a white callus one week after culture initiation, and the first somatic embryo appeared 1 month later. *C. madurensis* explants regenerated somatic embryos under all the hormonal conditions tested. However, an effect of media composition was observed. Actually, when the explants were incubated in the presence of 12 μ M of phenylurea-derivatives, the percentage of responsive explants was significantly higher as compared to BAP and HF conditions. Among the diphenylurea analogous compounds, the 2,3-MDPU showed the highest percentage of responsive explants (68.0 ± 0.3 , 49.3 ± 0.2 and 43.3 ± 0.2 in 2,3-MDPU, PBU and 4-CPPU, respectively). The control conditions gave lower percentages of responsive explants (33.7 ± 0.6 and 10.0 ± 0.1 in BAP and HF, respectively). Also the mean number of somatic embryos per embryogenic explant obtained with 2,3-MDPU (about four embryos/explant) was significantly higher from that obtained with HF, BAP and the other phenylurea derivatives (0.6 ± 0.1 , 1 ± 0.1 , 2.1 ± 0.6 and 1.2 ± 0.2 in HF, BAP, PBU and 4-CPPU, respectively). The mean percentage of embryo germination ranged from 70 to 80, with no significant differences among treatments and the plantlets were 10 cm high three months after embryo germination.

Ten ISSR primers and six 10-mer RAPD primers were used to amplify all of the regenerated plantlets under the five different hormonal conditions and the mother plant. A total of 190 reproducible and well-resolved band classes have been achieved and 26,974 bands (number of plantlets analysed \times number of common band classes with all the used primers plus the polymorphic bands) were generated using both the RAPD and ISSR techniques.

The ten ISSR primers used in this analysis gave rise to 122 well-resolved band classes, ranging from 200 bp [with

the primer (GA)₈YG] to 2.5 kb in size [with the primer (CA)₈RG]. The primer (GA)₈YG amplified the greatest number of bands (15), whereas the primer (AC)₈YA amplified the lower number of bands (9), with an average of 12 bands per ISSR primer.

The RAPD primers produced 68 reproducible band classes, ranging from 300 bp (with the primer OPN14) to 2.6 kb in size (with the primer OPM04). The number of RAPD bands obtained for each primer varied from 7 (with the primer OPN14) to 17 (with the primer OPH04), with an average of 11 bands per primer. A summary of the effectiveness of ISSR and RAPD markers, including the polymorphic scored fragments used in the data analysis, is given in Table 1.

Of 2,320 total profiles (total number of plantlets analysed \times total number of primers used) obtained, only four appeared different. Only one plant showed genetic variation among the plants obtained from somatic embryogenesis induced by the 2,3-MDPU diphenylurea derivative. This variability was due to one specific 1,850 bp fragment produced from the RAPD primer OPN14 that was present only in the regenerant R1, and absent in the mother plant (Fig. 1a, lanes 3 and 2, respectively).

In the same way, only one plant showed somaclonal variation among the plants regenerated in presence of PBU. In this case, the variability was due to one specific 980 bp fragment produced from the RAPD primer OPAT14 present in the regenerant R16, and absent in the mother plant (Fig. 1b, lanes 18 and 2, respectively).

Two plants regenerated in present of 4-CPPU showed genetic polymorphism. Only the regenerant R16 showed a 800 bp polymorphic fragment with the primer (AC)₈YA that was absent in the mother plant (Fig. 1c, lanes 18 and 2, respectively), and only the regenerant R5 showed a unique 860 bp polymorphic fragment with the primer (TCC)₅RY absent in the mother plant (Fig. 1d, lanes 7 and 2, respectively).

Thus among the somaclones regenerated in presence of 2,3-MDPU cytokinin, only 3.7% showed genetic variability. In the same way, the 3.7% of the somaclones regenerated using PBU were polymorphic. An higher potential of induction of somaclonal variability (7.4%) was observed in plants regenerated in presence of 4-CPPU cytokinin. No somaclonal variability was observed when an HF or BAP media were used.

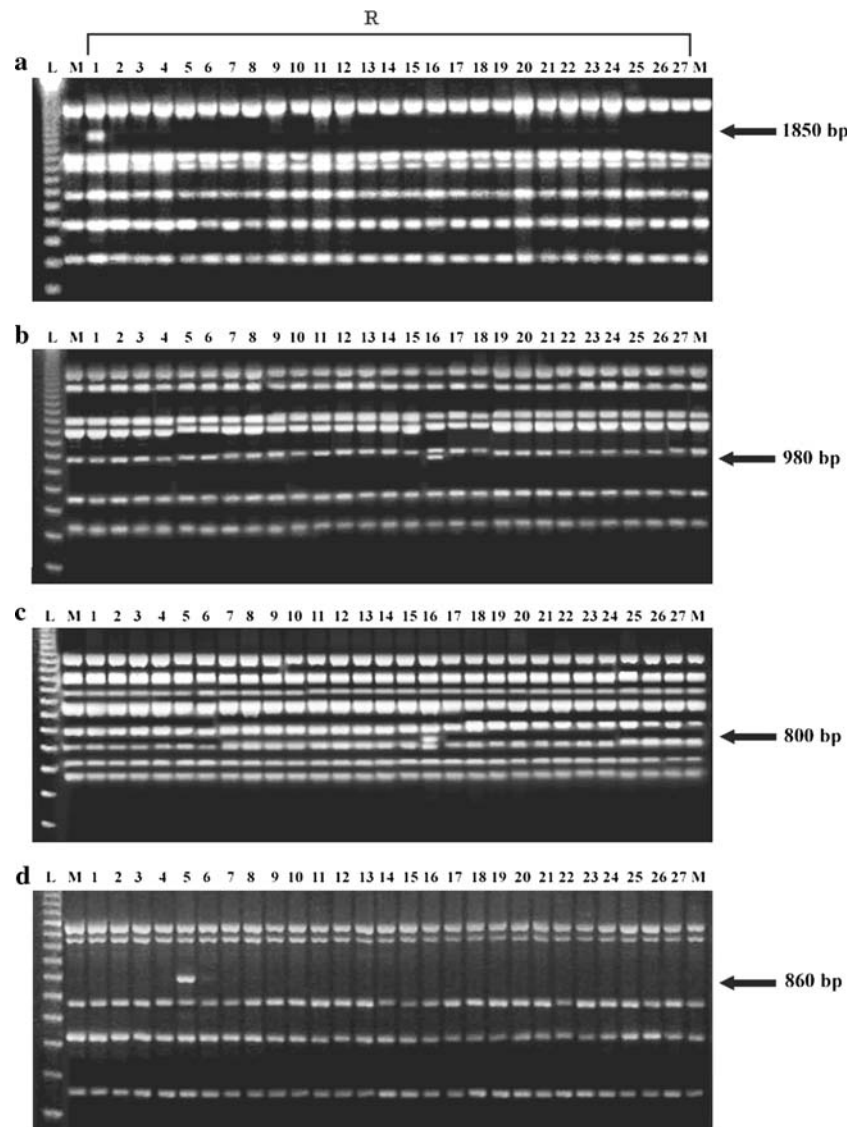
The percentage of polymorphism (P) identified was 1.05 when 4-CPPU was used, whereas it was reduced to 0.53 when 2,3-MDPU and PBU were used. The 4-CPPU was able to induce an increase of the total Nei's genetic diversity (H_T) of 0.0008 ± 0.0001 , twice greater than 2,3-MDPU and PBU ($H_T = 0.0004 \pm 0.0001$). All the mutated somaclones showed a genetic profile equally distant from the mother plant and all the not-mutated offspring, having

Table 1 Effectiveness of ISSR and RAPD markers in detecting polymorphisms of calamondin plants regenerated by somatic embryogenesis

	ISSR	RAPD
Number of primers used	10	6
Total band classes scored	122	68
Polymorphic fragments scored	2	2
Percentage of polymorphic primers	20.0	33.3
Percentage of polymorphism (P)	1.6	2.9
Minimum number of fragments per primer	9	7
Maximum number of fragments per primer	15	17
Average number of fragments per primer	12	11

with both a genetic distance (GD) of 0.0053. The genetic distances among the mutated somaclones were in all cases 0.0106.

Fig. 1 DNA profiles amplified from somaclones regenerated in different media. **a** Somaclones regenerated in media supplemented with 2,3-MDPU analysed with the RAPD primer OPN14; **b** somaclones regenerated in media supplemented with PBU analysed with the RAPD primer OPAT14; **c** and **d** somaclones regenerated in media supplemented with 4-CPPU analysed with the ISSR primers $(AC)_8YA$ and $(TCC)_5RY$, respectively. Lanes 3–29 regenerants, *L* 123-bp DNA ladder, *M* mother plant



Discussion

The embryogenic potential of three diphenylurea derivatives (2,3-MDPU, PBU and 4-CPPU) on calamondin style-stigma explants was investigated and compared with the adenine-derived cytokinin BAP and HF medium. We observed that in our experimental conditions the percentages of responsive explants obtained in presence of diphenylurea derivatives were significantly higher than that obtained with BAP and HF conditions. However, the DNA analysis of plantlets regenerated in presence of the diphenylurea-derived cytokinins, has showed an induction effect of somaclonal variations for these compounds as compared to the plants regenerated in control media HF and BAP. Until now, few studies on the incidence of somaclonal variation at morphological, cytological, biochemical and molecular levels have been carried out in *Citrus*. Some researchers

had reported having off-types of *Citrus* in plants regenerated in vitro through organogenesis or somatic embryogenesis. Navarro et al. (1985) observed that 29% of plants obtained by somatic embryogenesis from nucelli of monoembryonic *Citrus* presented abnormal phenotypic characteristics, whereas those regenerated by nucelli of polyembryonic *Citrus* cultivars were normal. The authors were not able to explain the different behavior of monoembryonic and polyembryonic cultivars. It is possible that the mutations, observed by Navarro et al. (1985), were probably already present in the nucelli at the time of explanting (Litz and Jaiswal 1991). Other reports on limited populations of polyembryonic *Citrus* species regenerated by somatic embryogenesis showed uniformity, at least with respect to those characteristics examined (Vardi and Spiegel-Roy 1982; Kobayashi 1987). Plants regenerated from *C. acida* Roxb. calli of different ages showed no changes in either morphology or in chromosome number (Chakravarty and Goswami 1999). More complete investigations on morphology, ploidy level, and leaf isozyme analysis on *Citrus* and *Citrus* hybrids have revealed no permanent variations among the plants regenerated through organogenesis or somatic embryogenesis (Gmitter et al. 1992). However, other studies on ‘Hamlin’ and ‘Valencia’ sweet orange coming from different in vitro culture methods showed significant stable variations of fruit characteristics, maturity date and ploidy level (Grosser et al. 1997, 2003).

Two different DNA-based techniques, RAPD and ISSR, were used for detecting genetic variations in calamondin-regenerated plants. We observed somaclonal variations in plantlets derived from somatic embryos regenerated in presence of diphenylurea derivatives, whereas we never observed somaclonal variations in plants regenerated in presence of the adenine-derived cytokinin (BAP) or in growth regulator free medium (HF). In our experimental conditions, the DNA analyses revealed the highest somaclonal variability when the 4-CPPU was used (7.4% of mutated plants). However, we could observe a much higher alteration at the phenotypic level in the plants growing in the field. This study supports that the use of two different types of molecular markers, which analyse different regions of the genome, permits a better and deeper analysis of genetic variations, as previously recommended in several somaclonal variability studies (Palombi and Damiano 2002; Martins et al. 2004).

It is already known that diphenylurea-derived cytokinins perform their hormonal functions in an indirect manner. Actually, more than functioning as canonical cellular hormones, they inhibit the cytokinin-oxidase (CKOx) enzymes resulting in an overall increase of the endogenous cytokinin levels (Burch and Horgan 1989; Victor et al. 1999). The CKOx are the only cellular enzymes devoted to the degradation of cytokinins. Seven of these have been identified

in *Arabidopsis thaliana*, carrying out a fine regulation of intracellular cytokinin levels (Werner et al. 2003).

It is known that high levels of cytokinins induce an increase of reactive oxygen species (ROS) levels in plant cells (Carimi et al. 2005). These molecules may react with a wide spectrum of metabolites, proteins and nucleic acids causing several cellular damages and inducing defensive responses (Gille and Siegler 1995). Oxydised enzymes are degraded by activated cytosolic proteinases (Laval 1996); the cell redox potential is altered (Cassells and Curry 2001) and, in the extreme case, cell death occurs (Hippeli and Elstner 1996; Polyak et al. 1997). Moreover, the potential of ROS to cause oxidative damage to both nuclear and organellar DNA (genotoxicity, Wiseman and Halliwell 1996) is significant. It can result in genomic changes expressed in altered hyper- and hypomethylation (Cerda and Weitzman 1997; Wacksmann 1997), aberrations of chromosome number from polyploidy to aneuploidy, chromosome strand breakage and rearrangements, DNA base deletions and substitutions (Gille et al. 1994; Czene and Harms-Ringdahl 1995; Hagege 1995). All these changes have been already considered as a possible explanation of wide range of genetic and epigenetic somaclonal variability found in plant becoming from cell, tissue and organ cultures (Cassells and Curry 2001). It is possible that the cytokinin-oxidase inhibition caused from the diphenylurea-derived hormones induces high cytokinin levels leading to ROS accumulation that, probably, are responsible for genetic mutations.

Recently, some researches demonstrated that high dosage of BAP and other cytokinins are responsible for nitric oxide (NO) synthesis in *Arabidopsis*, tobacco and parsley (Tun et al. 2001) and it occurs in a dose-dependent manner (Carimi et al. 2005). NO has been shown to generate mutations and DNA damage (Lin et al. 2000; Phoa and Epe 2002). Thus, the somaclonal variation observed in plants regenerated in the presence of the diphenylurea-derived cytokinins could be induced by high levels of ROS and/or NO.

To our knowledge, this is the first report investigating the somaclonal variability potential of synthetic phenylurea derivatives. Here, our results provide preliminary evidence for a role of phenylurea-derived cytokinins 2,3-MDPU, PBU and 4-CPPU in inducing somaclonal variations, and warrant further investigation. All these facts should be taken into consideration when new protocols for clonal propagation of the plants are being developed. Actually, somaclonal variations could represent a very serious problem for preserving the genetic integrity of the regenerated plants. On the other hand, the regeneration of somatic embryos in presence of diphenylurea derivatives, that induce somaclonal variability, could contribute to plant

genetic improvement programs. It should be interesting to clarify the role of ROS and NO on the somaclonal variability in plants regenerated in vitro in presence of diphenylurea derivatives.

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