

Full Length Research Paper

Enhancement of somaclonal variations and genetic diversity using graphite nanoparticles (GtNPs) in sweet potato plants

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To assay the efficiency of graphite nanoparticles (GtNPs) in sterilizing tissues and their role in enhancing genetic diversity, sweet potato is considered an important crop; hence its explants were used. In this experiment, GtNPs of 200, 400 and 800 ppm concentrations were used for sterilization of MS callus induction and regeneration media in Abees cultivar. The results showed that GtNPs had a good potential for removing bacterial contaminants without having side effects on the explant viability during the sterilization of sweet potato tissue in all their concentrations. Also, the percentage of callus induction increased from 98.67% in control to 100% in all GtNPs concentrations. The number of shoots per callus was enhanced at 400 ppm concentration. RAPD molecular markers and SDS-PAGE analysis were used to assess the genetic diversity of the sweet potato selected plants obtained from somaclonal variations in combination with GtNPs. Five decamer random amplified polymorphic DNA (RAPD) primers generated a total of 96 DNA fragments from the selected variants and their parent. Out of them, 82 polymorphic bands appeared with 85.42% polymorphism. The levels of DNA and protein patterns polymorphism within each treatment varied. RAPD and protein markers revealed that the concentration of 800 ppm showed the lowest similarity average among the ten selected variants and their parent. The obtained results indicated that somaclonal variation with GtNPs can be combined to increase the induced mutations frequency.

Key words: Graphite nanoparticles, somaclonal variation, random amplified polymorphic DNA (RAPD), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Sweet potato [*Ipomoea batatas* (L.) Lam], belonging to Convolvulaceae family, is a very important crop in the world. It is a good source of proteins, minerals, vitamins and antioxidants (Pfeiffer and McLafferty, 2007; Bovell-Benjamin, 2007; Tumwegamire et al., 2011). Due to its

commercial importance, the genetic improvement of the plant is needed. The essential way to improve this important crop is through the induction of genetic variations, which can be done by biotechnological interventions such as tissue culture. Using plant tissue

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culture makes the production of secondary metabolite, genetically modified and disease-free plants possible (Murashige, 1974; Khosroushahi et al., 2006). In spite of the advantages of tissue culture technique, some methodological problems, such as microbial contamination of explants hinder its importance as an advanced technique for biotechnological research (Cassells, 1991).

In modern science, material nanoparticles (NPs) display completely new or enhanced properties based on their size, distribution and morphology. Scientists suggested positive and negative effects of NPs on plants' growth and development. Many morphological and physiological changes can appear as a result of the interaction between nanoparticles and plants. The chemical composition, concentration, size and physical properties of NPs can determine their efficiency on plants (Ma et al., 2010; Khodakovskaya et al., 2012). Nanoparticles application led to the induction of microbe-free explants and demonstrated the positive role of NPs in callus induction, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation and secondary metabolite production (Kim et al., 2017).

Genetic variation resulting from *in vitro* culture, somaclonal variation is considered to be very useful for developing transgenic plants with desirable agronomic traits (Gaafar and Saker, 2006). Detecting genetic variation of transgenic plants is one of the purposes and criteria for their safety assessment. Random Amplified Polymorphic DNA (RAPD) marker has been used successfully as a molecular marker to characterize, identify and determine variations in nuclear genome between sweet potato genotypes (Gichuki et al., 2003; He et al., 2006; Lin et al., 2009; Moulin et al., 2012; da Silva et al., 2014; Galal and El Gendy, 2017). Also, this technique proved to be able to detect variation among individuals and to estimate the genetic diversity of somaclonal variations in various plant species (Hernandez et al., 2007; Sheidai et al., 2008; Khan et al., 2011; Nasim et al., 2012) including sweet potato (Aboulila, 2016).

Among biochemical markers, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a useful and inexpensive tool for describing genetic structure of several plant species (Oppong-Konadu et al., 2005; Salimi, 2013). Because of the importance of plant cell and tissue culture, and to avoid contamination, a dominant barrier in this technique, the aim of this work was to discover the effect of graphite nanoparticles (GtNPs) as antimicrobial agents and their role in enhancing somaclonal variations and diversity in sweet potato plants. Genetic diversity was assessed using RAPD molecular markers and SDS-PAGE analysis.

MATERIALS AND METHODS

This study was conducted at the Laboratories of Genetics

Department, Faculty of Agriculture, Kafrelsheikh University, Egypt.

Plant material

Greenhouse-grown plants of sweet potato, Abees cultivar, were considered as the experimental materials *in vitro* using nodal cutting system.

Nano material

Graphite nanoparticles (purity 99.9%) and particle size of 1 to 2 nm) were applied in the present study. GtNPs were diluted in double distilled water at concentrations of 200, 400 and 800 ppm and suspended by sonication for 30 min before use.

Preparation and characterization of GtNPs

GtNPs were prepared using the expanded graphite (EGt) method as described by Yu and Qiang (2012). One gram of EGt was immersed in 1000 mL aqueous solution of 75% alcohol and suspended by sonication for 12 h. GtNPs were purified using a filtration process. They were washed with distilled water and then allowed to dry in a thermo-static vacuum oven at 100°C. Physical characterization and diameters of nanoparticles were noticed and measured by a transmission electron microscopy (TEM). The result of the TEM image of GtNPs (Figure 1) showed that the particle sizes are in the range of nano.

GtNPs treatments, callus induction and regeneration system

To study the effect of GtNPs on callogenic response and antimicrobial effects, stem segments without buds were surface disinfected with 70% EtOH for 30 s, 2.5% NaOCl for 5 min and 0, 200, 400 and 800 ppm GtNPs for 15 min before they were used as explant materials. Explants were cultured on MS (Murashige and Skoog, 1962) medium provided with sucrose (30 g/L), BAP (8 mg/l), myo-inositol (100 mg/l) and three different concentrations of GtNPs (200, 400 and 800 ppm) besides the control. The final pH value was adjusted to 5.8 and the media were solidified with 2 gm/l phytagel. Each treatment consisted of three replicates (five Petri dishes with five explants for each replicate). The explants were incubated at 25±2°C in darkness for 6 weeks with two sub-culturing. Six days after culturing, callusgenesis was started. Explants were checked daily for any possible contamination. At the end of callus induction period, microbial contaminants were recorded. Moreover, callus induction percentage was estimated as the percentage of explants that produce callus.

Calli obtained from each treatment were subsequently transferred to shoot induction medium comprising complete MS medium with 100 mg/l myo-inositol and 6.0 mg/l BAP (containing GtNPs graded levels). The observations were recorded after two weeks of incubation for microbial contaminants, percentages of shoot induction (based on number of calli forming shoots) and the number of shoots/callus.

Hardening of *in vitro* plantlets

Regenerated shoots (3-5 cm in length) were excised from the embryogenic callus, transformed and cultured on half strength MS medium; their pH was adjusted to 5.8. Cultures incubation was done in growth room and maintained under conditions previously mentioned. For acclimatization, the obtained plantlets were hardened as described by Aboulila (2016).

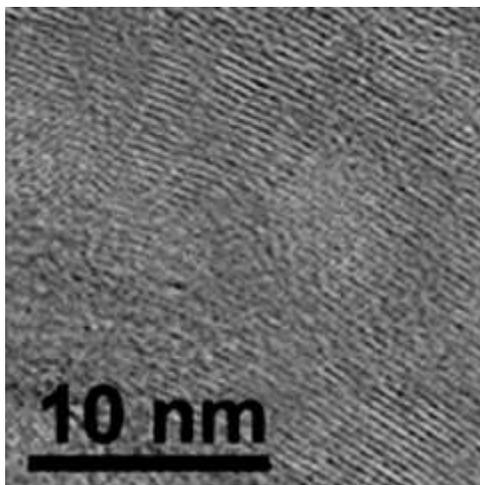


Figure 1. TEM image of graphite nanoparticles.

Table 1. Sweet potato culture response to callus induction and plant regeneration media.

Treatment (ppm)	Callus induction (%)	Shoot induction (%)	Number of shoots/callus	Microbial contamination
Control	98.67±1.33 ^a	100 ^a	8.67±0.53 ^b	(+)
200	100 ^a	85.67±4.25 ^b	7.87±0.56 ^b	(-)
400	100 ^a	96.33±1.53 ^{ab}	13.73±1.85 ^a	(-)
800	100 ^a	63.11±8.74 ^c	6.67±0.83 ^b	(-)

Molecular analysis

Total genomic DNA was isolated from fresh leaves of the parent plant and ten selected somaclonal variants of each of the control and the three GtNPs treatments using the method of CTAB-chloroform as described by Saghai-Marouf et al. (1984). Random amplified polymorphic DNA analysis using five oligonucleotide decamer primers was applied; OPA-20, OPB-01, OPB-05, OPB-07 and OPB-17 (Bio Basic Inc, Canada). The PCR reaction mixture consisted of 0.75 µl of genomic DNA (40 ng), 0.75 µl of 20 µM primer, 5 µL of 2X PCR Master mix Solution (i-Taq™, iNtRON's Biotechnology) and 3.5 µL of sterile distilled water in a final volume of 10 µl. Amplification condition was performed according to Galal and El Gendy (2017). Amplification products were separated by electrophoresis and bands were detected on Benchtop UV-transilluminator and photographed using Doc-It™ Imaging System. A known 50 bp DNA Ladder ready-to-use (Cat-no: 300003, GeneON) was run against the PCR products.

Biochemical analysis

Total soluble proteins were obtained from 0.5 g fresh leaves of all selected somaclones with their parent. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for total soluble protein was done using 12.5% polyacrylamide gel as described by Laemmli (1970). Molecular weights (MW) for all obtained bands were determined by using pre-stained high molecular weight standard marker (PINK Prestained Protein Marker, Cat. No. MWP02), with molecular weights ranging from 15 to 175 kDa.

Data analyses

Data of recorded traits were analyzed statistically as complete randomized design in three replicates (n=5); the mean values obtained from the treatments were compared by the least significant differences (LSD) test at significance level of $P \leq 0.05$ using the SXW program.

Molecular and biochemical data were introduced to SPSS package program as: Binary value of 1 for visible band and 0 for absent band; genetic similarity was estimated using Jaccard's similarity coefficient (Jaccard, 1901).

RESULTS AND DISCUSSION

Effect of GtNPs on tissue culture

The influence of different concentrations of GtNPs (0, 200, 400 and 800 ppm) was evaluated by adding these concentrations to callus induction and shoot induction media. Results in Table 1 and Figure 2 show that regeneration capacity (shoot induction % and number of shoots/callus) was affected by the concentration of GtNPs. Callus induction percentage did not differ significantly in all treatments, which varied from 98.67% in control to 100% in all GtNPs concentrations. Also, in control treatment all of the calli were regenerable (100%)

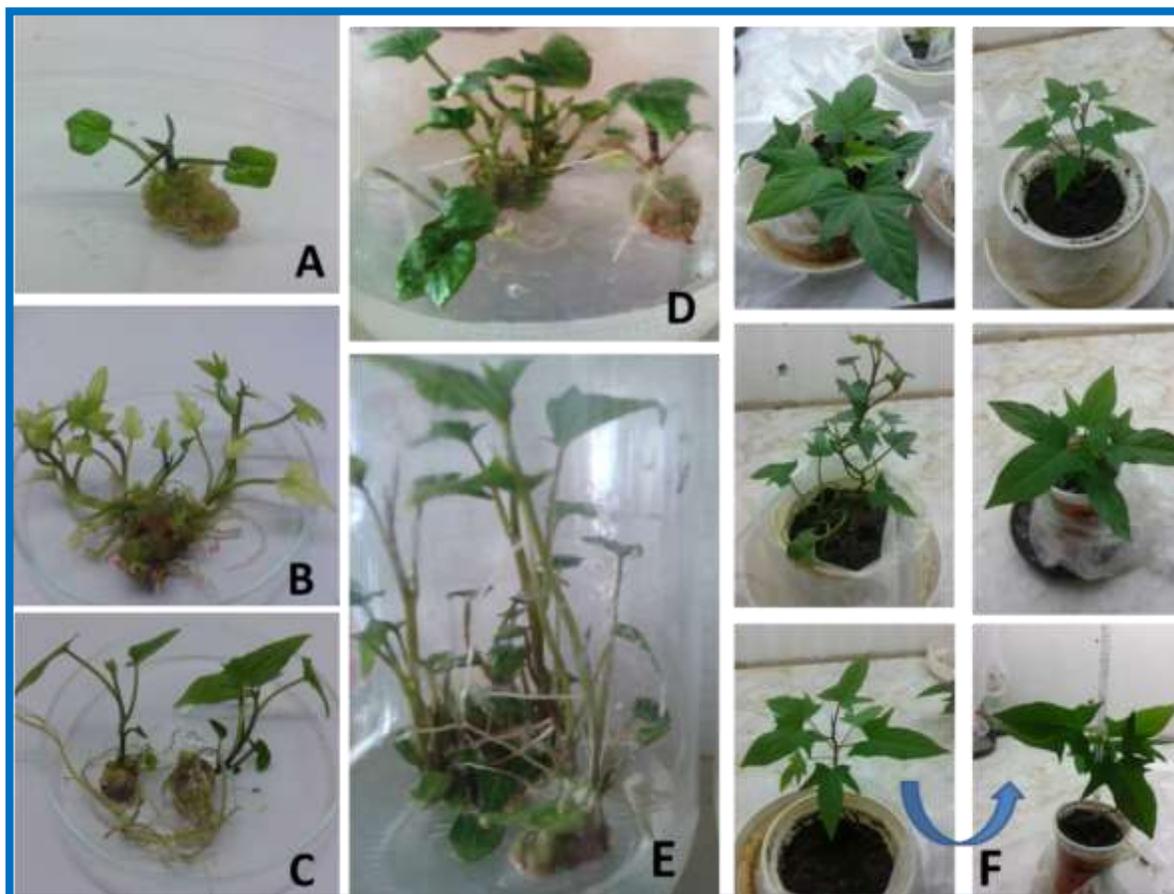


Figure 2. Callus induction and plant regeneration from Abees cultivar. (A) Callus induction; (B) shoot induction; (C) rooting; (D) shoot subculture; (E) shoot multiplication and elongation; (F) hardening of regenerated plants with different phenotypes.

followed by 400 ppm concentration which regenerated 96.33% from all cultured calli on regeneration medium. Plant regeneration and production of multiple shoots from callus were obtained after four to five weeks from callus initiation. While 400 ppm GtNPs recorded the highest number of shoots per callus (13.73) in regeneration medium, the other treatments recorded regeneration capacities ranging from 6.67 to 8.67. These results agree with those of Lahiani et al. (2016) who used carbon-based nanomaterials (CBNs) on tobacco cell culture to increase growth (22-46%) by addition of 50 $\mu\text{g/l}$.

Moreover, Khodakovskaya et al. (2012) reported that the incorporation of 100 mg/l multi-walled carbon nanotubes into a medium containing 1 mg/l 2,4-D increased callus growth of tobacco explants (64% increase over control). The treatment with carbon nanotubes enhanced callus growth by upregulation of cell division genes (CycB) and water transport (NtPIP1). However, carbon nanotube treatment (10-600 mg/l) decreased cell viability and dry weight in *Arabidopsis* (Lin et al., 2009). Hence, from all of these reports it can be summarized that the addition of NPs to a plant tissue

culture medium affects callus proliferation, shoot multiplication, somatic embryogenesis and rooting by altering antioxidant enzyme activities, gene expression and production of ROS.

Changes observed in developed organs and plantlets are termed somaclonal variation. In this study, some of the obtained regenerated plants after adaptation showed morphological differences compared to their parent, Abees cultivar (Figure 2F). These morphological differences are usually associated with changes in chromosome number, chromosome structure, DNA sequence, DNA methylation and mitotic crossing over (Bairu et al., 2011; Sivanesan and Jeong, 2012).

Positive effects of nanoparticles appeared on callus induction, shoot regeneration and growth in several studies. Aghdai et al. (2012) found that shoot induction percentage, number of shoots and callus formation increased when culturing the stem explants from *Tecomella undulata* (Roxb) on MS media containing 10 mg/l AgNPs. NPs treatments affect the mitotic index and DNA integrity, and alter the protein and DNA expression in plants (Atha et al., 2012; Landa et al., 2015; Tripathi et

al., 2017).

Identification of anti-microbial effect of GtNPs

The efficiency of GtNPs in decontaminating diverse kinds of explants was evaluated. So, we used different concentrations of GtNPs to find the best sterilization treatment. Sweet potato explants were cultured on GtNPs and GtNPs-free media for callus induction and regeneration to study the elimination of microorganisms in all procedures of tissue culture after four weeks. The results showed that, treating sweet potato stem segments with 200, 400 and 800 mg/l GtNPs for 15 min, there were no contaminants with no effects on organogenesis. Moreover, contaminations were observed only in control treatment (GtNPs-free media) during the different stages in culture media, while microbial contaminants were absent in all GtNPs concentrations. These results showed that GtNPs were effective in the suppression of microbial contaminants in all concentrations.

When GtNPs of 200, 400 and 800 ppm concentrations were applied on sweet potato explant segments, they could function as antimicrobial agents leaving no harmful effect on explants and their viability, and were all able to produce callus. It has been illustrated that the toxicity of GtNPs to microbial cells is apparent even at the range of 200 to 800 ppm concentrations. Several types of NPs such as silver (Ag), aluminum oxide (Al_2O_3), copper oxide (CuO), iron oxide (Fe_3O_4), gold (Au), magnesium oxide (MgO), nickel (Ni), silicon (Si), silicon dioxide (SiO_2), titanium dioxide (TiO_2), GtNPs and zinc oxide (ZnO) have been reported to possess antimicrobial activities against various microorganisms (Liu et al., 2011; Gouran et al., 2014; Beyth et al., 2015).

Genetic diversity in sweet potato using RAPD and protein markers

Genetic polymorphism using RAPD analysis

Five oligonucleotide random primers were employed to study the genomic stability in sweet potato parental cultivar (Abees) along with its somaclonal variants obtained from tissue culture combined with GtNPs (Figure 3). Among the primers used, OPB-17 generated the highest number of total bands (27 bands), while primer OPB-05 produced the lowest (13 bands). All primers generated a total of 96 DNA bands. Fourteen bands were monomorphic and consistent among all selected variants for all treatments; however, 82 bands were observed to be polymorphic with 85.42% polymorphism. The highest level of polymorphism (96.30%) was recorded in primer OPB-17 while the lowest level of polymorphism was 72.22% in primer OPB-07 (Table 2).

On the other hand, the five primers revealed a total of 93 bands in the parental genotype. Out of them, 52, 58, 35 and 47 bands were common in the parental genotype and the somaclonal variants obtained from the four treatments; control, 200, 400 and 800 ppm, respectively (Table 3). The level of polymorphism among the somaclonal variants and the parental genotype varied. The highest number of polymorphic bands (61 bands) was recorded in 400 ppm concentration with 63.54% polymorphism, while the lowest number (37 bands) was noticed in 200 ppm with 38.95% polymorphism. The genetic polymorphism increased in the regenerated plants at 400 and 800 ppm, while it decreased at 200 ppm compared to the control.

Variations observed in the total number of RAPD bands among the parental genotype and plants generated from tissue culture combined with different GtNPs concentrations indicate genetic differences of the variants because of somaclonal variation induced from tissue culture as seen in control treatment, plus the genetic variation induced by GtNPs treatments. This is in agreement with Sheidai et al. (2008) who found that some bands appeared in the parental plants and got lost in regenerated plants because of somaclonal variation. These results proved that RAPD markers were effective in detecting polymorphism which occurs due to insertion, deletions and base substitution that affect the primer-binding site and reflect as the presence or loss of bands. These findings are in agreement with earlier studies using RAPD analysis in describing genetic polymorphism among somaclonal variants in various plant species. Khan et al. (2011) used this technique to determine the genetic variations among micropropagated banana plants.

Genetic polymorphism based on SDS-PAGE analysis

Protein banding patterns were used to detect the genetic variations among the ten selected variants within the four studied treatments (Figure 4). The electrophoretic patterns of SDS-protein revealed marked polymorphism within each GtNPs treatment as shown in Table 3. Polymorphism percentage within the four treatments ranged from 33.33 to 100%. Control treatment revealed the lowest polymorphic percentage (33.33%) indicating that level of polymorphism differed a little within the control treatment. Also, 800 ppm treatment gave the highest polymorphic percentage (100%); a total of nine bands ranging from 2 to 9 were detected and all of them were polymorphic. For the other two treatments (200 and 400 ppm), the profile of variants and their parent exhibited the highest number of protein bands (10 bands for each) which showed 90 and 80% polymorphism, respectively.

Results obtained from many studies have shown that much of the genetic variability generated from plant

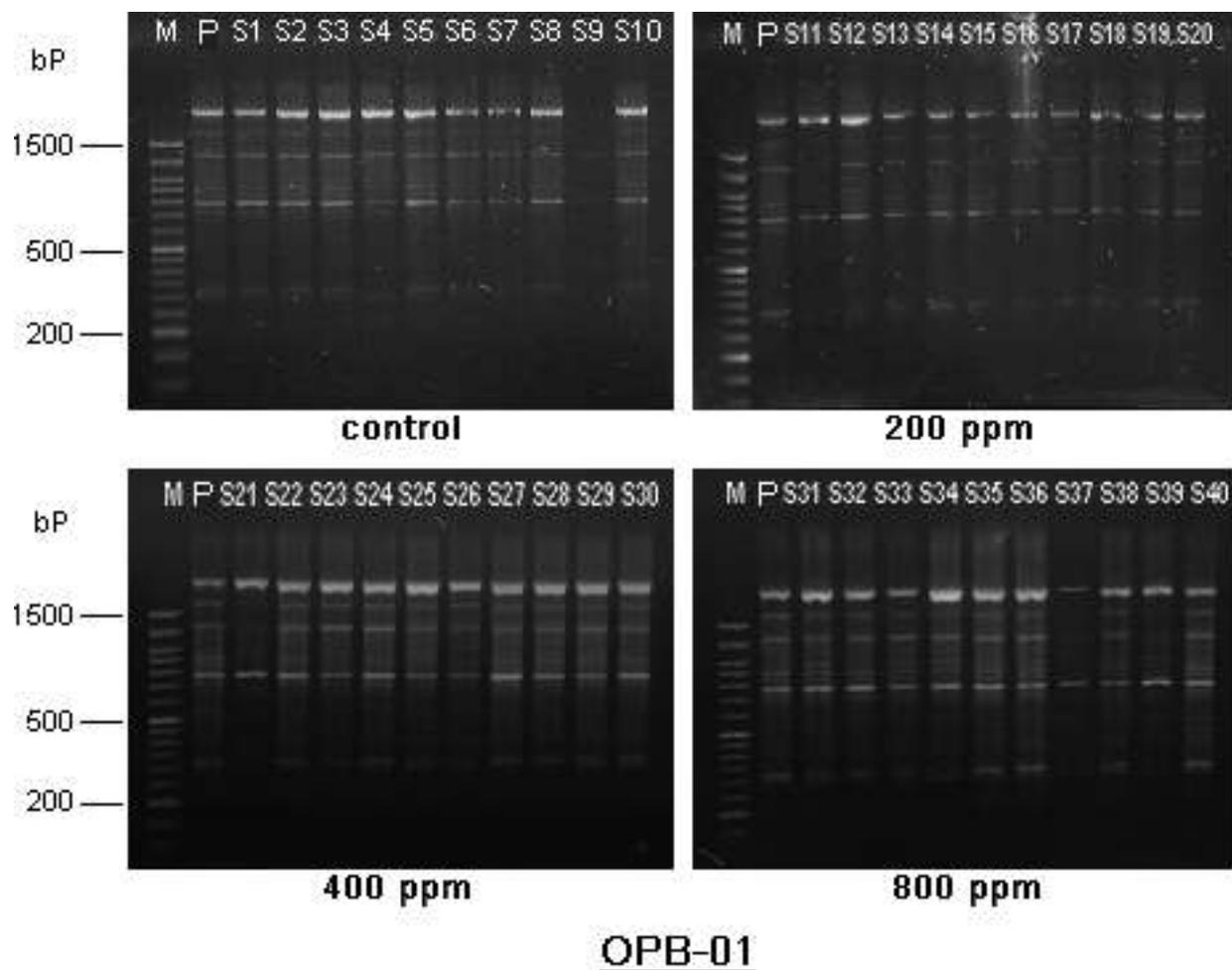


Figure 3. RAPD profile of OPB-1 primer. P, parental plant; S1-S10, S11-S20, S21-S30 and S31-S40: somaclonal variants of the four GtNPs (control, 200, 400 and 800 ppm, respectively). L, 50 bp molecular ladder.

Table 2. Level of polymorphism among the parental genotype and the somaclonal variants treated with different concentrations of GtNPs, based on RAPD analysis.

Primer name	Sequence (5'→3')	TAF	MB	PB	P (%)	AF				
						Parent	Treatments (10 variants)			
							control	200 ppm	400 ppm	800 ppm
OPA-20	GTTGCGATCC	15	4	11	73.33	15	11-15	11-15	7-15	7-15
OPB-01	GTTTCGCTCC	23	1	22	95.65	23	2-23	12-21	13-23	12-23
OPB-05	TGCGCCCTTC	13	3	10	76.92	13	10-13	11-13	6-13	10-13
OPB-07	GGTGACGCAG	18	5	13	72.22	17	12-18	15-18	16-18	11-17
OPB-17	AGGGAACGAG	27	1	26	96.30	25	22-26	22-26	3-25	20-25
Total	-	96	14	82	85.42	93	95	95	96	94

TAF, Total amplified fragment; MB, Monomorphic bands; PB, Polymorphic bands; P (%), Polymorphism (%); AF, Amplified fragment.

tissue culture may be the result of gene mutation (D'Amato, 1985; Ngezahayo et al., 2007) or epigenetic variation (Kaepler et al., 2000; Guo et al., 2006;

Smulders and de Klerk, 2011). It is likely that these variations are based on the differences in GtNPs concentrations, media used for culture or their

Table 3. Polymorphism percentages generated by RAPD and protein markers within the ten somaclonal variants obtained from each of the four GtNPs treatments.

Treatment (ppm)	TB	Range of bands (ten variants)	MB	PB	P (%)
RAPD markers					
Control	95	69 - 92	52	43	45.26
200	95	79 - 91	58	37	38.95
400	96	68 - 92	35	61	63.54
800	94	66 - 86	47	48	51.06
Protein marker					
Control	6	4 - 6	4	2	33.33
200	10	3 - 10	1	9	90.00
400	10	3 - 8	2	8	80.00
800	9	2 - 9	0	9	100

TB, Total bands; MB, monomorphic bands; PB, polymorphic bands; P (%), polymorphism (%).

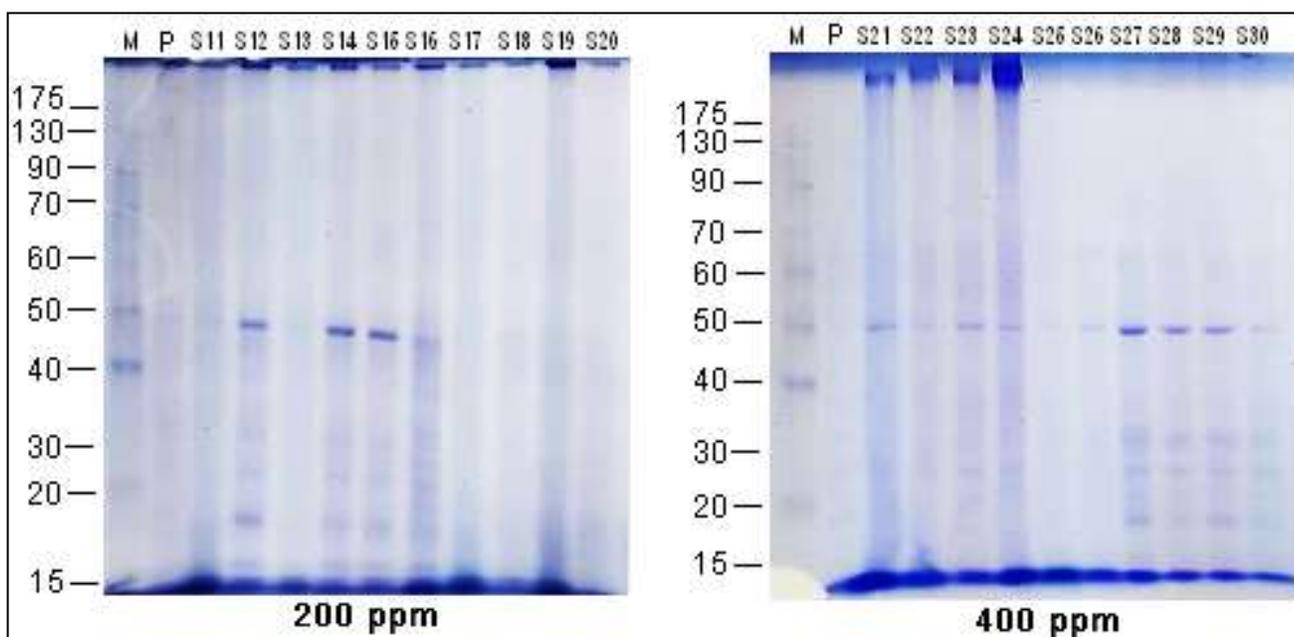


Figure 4. The protein banding patterns of the parental genotype (P) and the ten somaclonal variants (S11-S20 and S21-S30) treated with 200 and 400 ppm of GtNPs, respectively. M, Protein molecular marker from 15 to 175 KDa.

combinations. This is in contrast with the results of Afrasiab and Iqbal (2010), who noted that the recovery of somaclones can be increased by combining micro-propagation with induced mutagenesis *in vitro*.

Genetic similarity

The ranges and averages of similarity values for the ten somaclonal variants and their parent within each of the GtNPs treatments (200, 400 and 800) and control based

on RAPD and protein markers are listed in Table 4. RAPD and protein markers revealed that the concentration of 800 ppm showed the lowest similarity average among the ten selected variants and their parent. In the case of RAPD marker, all treatments revealed high similarity averages ranging from 0.816 (800 ppm) to 0.867 (200 ppm), with a mean value of 0.843, indicating high homogeneity within the tested treatments. While, the genetic similarity decreased with GtNPs increase from 200 to 800 ppm, indicating that genetic variations induced in the regenerated plants increase with

Table 4. Similarity ranges and averages within each of the four treatments based on RAPD and protein markers.

Treatment	RAPD marker		Protein marker	
	Range	Average	Range	Average
Control	0.690-0.968	0.864	0.667-1.00	0.850
200 ppm	0.766-0.946	0.867	0.167-1.00	0.563
400 ppm	0.575-0.978	0.825	0.286-1.00	0.605
800 ppm	0.656-0.925	0.816	0.00-1.00	0.416
Mean	0.575-0.978	0.843	0.00-1.00	0.608

the concentrations of GtNPs. Concerning protein marker, the similarity mean value of 0.608 was obtained from all tested treatments. The highest genetic similarity average was found within control treatment (0.850), while the lowest one was observed within 800 ppm concentration (0.416). However, 200 and 400 ppm treatments showed genetic similarity averages with 0.563 and 0.605, respectively.

Therefore, analyses of RAPD and SDS-PAGE appeared to be effective for assessing genetic similarity of sweet potato somaclonal variants and their parent within each GtNPs treatment. These results are in line with those of Metry et al. (2002) who used RAPD markers and SDS-PAGE analysis to identify genetic similarity among transgenic potato cultures.

Conclusion

In conclusion, results showed that genetic variations occurred due to the differences generated from somaclonal variation during tissue culture combined with that generated from GtNPs treatments. Somaclonal variations and GtNPs can be combined to increase induced mutations frequency. Mutation, which changes one or few specific traits of a cultivar, can be utilized for selection of desired traits in sweet potato to crop improvement. The obtained variants could be used for more evaluation to test their commercial biosafety.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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