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Research Article

Study of Molecular Diversity in *Celosia argentea* Cristata (L.) Mutants using RAPD Markers

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Abstract

Induced mutagenesis is a method of creating genetic variability in crops for its improvement. Mutant lines of Celosia argentea, irradiated with sodium azide and fast neutron were evaluated for genetic variability patterns. Mutant lines were collected from the Department of Plant Biology, Federal University of Technology, Minna and analysed using five RAPD primers at the Biosciences Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Data generated were subjected to UPGMA clustering and principal component analysis. Five RAPD primers generated 21 amplification bands, with all the bands being 100% polymorphic. Allelic frequency per primer ranged from 0.40 in Primer (OPH05, OPB17 and OPB04) to 0.70 (OPT17) with an average frequency of 0.48 per primer. The highest polymorphic information content (PIC) of 0.70 was generated by primer OPH05 with an average PIC of 0.55 per primer. The clustering of the mutants and their parent into five distinct genetic groups by UPGMA dendrogram and four groups by principal component analysis was not based on the mutagenic agent used confirming the high level of induced diversity among the treatments. Therefore, Sodium Azide and Fast Neutron Irradiation are effective mutagens for inducing useful variability in Celosia argentea.

Keywords: *Celosia argentea, Fast Neutron, Mutant Diversity, PCA, Sodium Azide*

Introduction

Mutation breeding has been reported as an alternative to the conventional method of crop improvement (Girija *et al*., 2013). Mutagenesis creates novel alleles that do not exist in nature germplasms; thereby resulting in variability among plants from which isolation of enhanced and unique genetic material could be made for the improvement of the crop plants (Abubakar *et al*., 2015; Wang and Wang, 2019). Gupta (2019) reported that mutagenesis and mutation breeding plays an important role in vegetable crop improvement, among which *Celosia argentea is* inclusive. *Celosia argentea* is an annual plant cultivated for its nutritional, medicinal and ornamental values in the Amaranthaceae family. The plant is a rich source of proteins, crude fibre, calories, vitamins and minerals; with the leaves containing a high level of calcium, phosphorus and iron (Law-Ogbomo and Ekunwe, 2011). In addition, it has high percentage of essential amino acids with methionine as the limiting amino acid (Ayodele and Olajide, 2011). Patel *et al*. (2010) reported that the curative properties of the plant are due to the presence of complex phytochemicals; alkaloids, glycosides, flavonoids, saponins and tannins in varied compositions as secondary metabolites.

Despite the nutritional importance and high demand for the leafy vegetable, the crop is faced with challenges of genetic erosion driven by cultural and environmental factors. Effort had been made to improve the crop through conventional and mutation (Akinloye *et al*., 2013; Mostafa *et al*., 2014; Abubakar *et al*., 2015) breeding methods and considerable successes had been reported. However, the genetic diversity of its mutants has not been well studied for the

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effective isolation of novel mutants endowed with new and desirable traits. Estimation of genetic diversity had been reported to be crucial for the determination of evolutionary relationships within and between species, as well as effective breeding strategies for a crop (Bhandari *et al*., 2017). Phenotypic characterisation alone had been reported not to be sufficient for the characterization of the available breeding material due to the tight relations between phenotype, growth conditions and cultivation techniques (Bahurupe *et al*., 2013). However, molecular markers present numerous advantages over conventional phenotype based alternatives; and DNA markers had proven to be efficient tools for the characterisation of the plant species (Daudu *et al*., 2016). Selected mutant varieties had been analysed at the molecular level using RAPD and the differences in variability determined (Girija *et al*., 2013). Estimates derived by dominantly inherited markers such as RAPD, AFLP, and ISSR are quite analogous and may be directly comparable (Nybom, 2004). Hence molecular diversity in *Celosia argentea* mutants using RAPD markers was carried out.

Materials and Methods

Collection of plant materials

Parent and Mutants' seeds of fast neutron irradiation (M_1 seeds generation of *Celosia argentea* exposed to fast neutron irradiation (FNI) from Americium Beryllium source (241Am/Be), of flux density 1.5 × 104n.cm-2S-1 for 30, 60, 90 and 120 min equivalents to 0, 4, 8, 12 and 16 µSv at the Centre for Energy and Research Training (CERT), Ahmadu Bello University, Zaria, Kaduna State, Nigeria) and sodium azide (M_1 seeds of 0, 2, 4, 6 and 8 millimolar [mM] concentration) were collected from the Department of Biological Sciences, Federal University of Technology, Minna and planted in properly labelled experimental pots at the Botanical of the Department. Fresh young (two weeks old) leaves were collected in properly labelled envelopes for each treatment, preserved with ice packed and transported to Biosciences laboratory, International Institute for Tropical Agriculture (IITA), Ibadan, Oyo State for molecular characterisation.

Molecular Characterisation

DNA extraction and quantification

DNA extraction was done on three (3) collected leaves samples using the RAPD method for the preparation of plant genomic DNA for PCR analysis described by Edwards *et al*. (1991) as modified by Daudu *et al*. (2016). Approximately 2.0 g leaves sample of each treatment was ground in liquid nitrogen and transferred into a 1.5 ml Eppendorf tube to which 700 µl of pre-heated plant extraction buffer was added. The samples were incubated for 20 minutes at 65°C and allowed to cool for 2 minutes. Ice-cold 5 M potassium acetate (500 µl) was added and incubated on ice for 20 minutes. A volume of 700 µl of chloroform-isoamyl alcohol mixture (24:1) was added to each sample tube and centrifuged at 12,000 rpm for 10 min to obtain protein and lipids. The supernatant was transferred into a new tube and 500 µl of ice-cold isopropanol was added, mixed gently and incubated at - 80^{o}C for 15 mins to precipitate the DNA. The DNA pellet was obtained by centrifuging the sample tubes at 12000 rpm for 10 mins and the supernatants were decanted to the last drop. The DNA pellet was washed by centrifuging in 100 µl of 70 % ethanol and the ethanol was decanted. Air-dried samples of the DNA were re-suspended in 60 µl of ultra-sterilised water into which 2 µl of RNase was added and incubated for 30 minutes at 37°C. The purity and quality of DNA were checked and quantified using 0.8 % Agarose and Nanodrop Spectrophotometer ND-1000. The extracted DNA samples were preserved at -20°C.

PCR amplification using RAPD primers

Five RAPD primer sets provided by IITA were screened and used for polymerase chain reaction (PCR). The PCR cycle was carried out with 9700 Applied Biosystems Thermal Cycler. Initial denaturation of the DNA template was carried out at 94^{o}C for 3 minutes followed by 45 cycles at 94^{o}C for 20 seconds. Annealing was carried at different levels at 37^{o}C for 40 seconds and 72^{o}C for 1 min and by extension at 72^{o}C for 7 minutes. The product was stored at 4^{o}C and amplified on 2 % agarose gel containing ethidium bromide stain at 80 volts electrophoresis for 4 hrs. The molecular fragments were estimated using 50 base pairs (bp) PCR markers. The gel

photographs of the probes were taken under UV light (Figure 1).

Data analysis

Scoring of the DNA bands generated by the primers was based on the degree of amplification of gel photographs. The presence of an allelic band was scored 1 and the absence of band zero (0) and binary matrix (0 or 1) was generated. The data obtained were subjected to Principal Component Analysis (PCA) to determine patterns of variation among the mutants. Principal components (PCs) with an eigen-value above one (1) were considered significant in determining variability among the mutants and component loadings greater than ±0.30 were considered to be meaningful (Hair *et al*., 2014). Dice Dissimilarity index was calculated between the lines using Darwin 5.0 software and a cluster diagram was drawn using Unweighted Pair Group Method Average (UPGMA) to determine the evolutionary relationship among the lines (Tamura *et al*., 2013).

Results and Discussion

Molecular characterisation of red variety of C. argentea

A total of 21 amplified bands were generated by five sets of primers of which the entire band were (100%) polymorphic. Primer OPH05 and OPB17 have the highest amplification of 6 bands each. The major allelic frequency varied from 0.40 in Primer (OPH05, OPB17 and OPB04) to 0.70 in primer OPT17 (Table 1). The highest genetic diversity (0.74) and PIC (0.70) were obtained in primer OPH05 while primer OPB17 had the lowest genetic diversity of 0.48 and 0.45 PIC. The average genetic diversity and the polymorphism information content (PIC) recorded were 0.62 and 0.55.

Dice dissimilarity index

The dissimilarity index values among the mutant lines showed the level of relatedness of the samples. Treatments 4mM and 8mM were identically the same with a dissimilarity index of 0.00 (Table 2). High dissimilarity values among the treatments ranged from 0.20 to 1.00. A high dissimilarity index (1.00) was obtained between the parent and buffer, 4mM, 8mM and 90 minutes irradiated mutants. The lowest dissimilarity value (0.20) was between buffer control and 4mM as well as 8Mm (Table 2).

Principal component analysis

The principal component analysis (PCA) revealed that the first eight principal components accounted for the entire (100%) variability among the mutants and the parent (Table 3). The eigen value of the component varied from 0.02 to 1.61. The first five (5) components explained 93.52% of the total variability among the mutants with the first component accounted for 42.15%. The variability in PC1 was mainly due to the contribution of all the treatments and PC2 was mainly due to buffer, 2mM, 4mM, 8mM and 90 minutes. The scatter plot of PC1 (42.15%) and PC2 (19.72%) revealed four clusters of inter-relationships among the mutants (A, B, C and D). However, the clustering of the mutants was not based on the type of mutagenic agent used for treatment of the mutants. Clustered B and D are composed of single mutant; 90 and 30 minutes, respectively (Figure 1). 4mM and 8mM are clustered as single mutants (100% the same) along with 90 minutes irradiated mutant and buffer (control) in group A while cluster C was composed of 2mM, 60, 120 minutes irradiated mutant and the parent stock.

Cluster analysis

Cluster analysis for the selected mutants and their parent genotype is presented in Figure 1. The cluster dendrogram generated two major clusters (A and B) and 5 distinct sub-clusters at a genetic distance of 3.0 (Figure 2). Cluster A was made of three sub-cluster (I, II and II) while Cluster B comprised sub-clusters IV and V. Sub-cluster II and IV are made up of single mutant 30 and 90 minutes irradiated mutants, respectively. Sub-clusters I and III were composed of two lines each; with the parent genotype and 120 minutes FNI clustered in sub-group I, while 60 minutes FNI and 2mM were clustered in III. Sub-cluster V was composed of 40% of the studied mutants (Buffer, 4mM, 6mM and 8mM) with 4mM and 8mM clustered as a single mutant.

Fast neutron bombardment had been known for causing translocations, chromosome losses, and large deletions while sodium azide mutagenicity

is mediated through an organic metabolite entering the cell nucleus, interacting with the DNA, creating point mutations in the genome. The 100 percentage polymorphism was obtained in all the RAPD primers used, indicating their capability in detecting alterations in the various primer binding sites of the mutants and suggesting their effectiveness in studying the genetic diversity of mutants *Celosia argentea*. Dhakshanamoorthy *et al*. (2011) reported that induced mutations contribute to genetic variability mainly by increasing DNA polymorphism. This result corroborated with that of Akinloye *et al*. (2013), who reported a high percentage of polymorphic bands in six varieties of *Celosia*. They attributed this to the fact that RAPD primers are highly polymorphic and could be useful in the estimation of genetic relationships among *Celosia*. In concordance with these reports, Hofmann *et al*. (2004) stated that the high RAPD polymorphisms observed among mutagenic embryogenic suspension cultures of soybean (*Glycine max* L. cv. Iroquois) using varying concentrations of ethyl methane sulfonate (EMS) could be due to alterations in the number of primer binding sites following mutagenesis. Point mutations at other regions of the genome that fall within the amplified fragments are likely to go undetected.

A higher number of alleles and high polymorphism have been reported to be important for the correct estimation of genetic diversity and the effectiveness of markers development (Pfeiffer *et al*., 2011). The high dissimilarity values recorded among the treatments might be attributed to the changes in the genetic composition of the plants that could occur due to the different doses or concentrations of the mutagens. Similarly, observation had earlier been reported by Dhakshanamoorthy *et al*. (2011), with high dissimilarity values recorded between all the treatments and parents of gamma irradiated *Jatropha curcas*, indicating a high level of genetic diversity among the samples. In agreement with these results, Taheri *et al*. (2014) reported that mutagens; both chemical and physical can interact with cellular molecules in the plant, particularly water, to produce free radicals (HOH) which combine to form toxic substances, such as hydrogen peroxide (H_2O_2). This brings about modification or damage of important cell components and affected the morphology, biochemistry, and physiology of plants (Wang and Wang, 2019). The possible genotypic differences among the mutant plants might have occurred based on the sensitivity of the irradiated tissue to mutagenic treatments (Ahloowalia, 1998).

The clustering of the mutants and the parent into 5 distinct groups by UPGMA analysis and 4 groups by PCA based on their genetic matrix is an indication of high genetic diversity among the treatments. This confirms the statement that mutants were differentiated genotypically, based on the occurrence of new bands and disappearance of old bands in combined random amplified polymorphic DNA (RAPD) profiles (Khan *et al*., 2012). Also, similar to the observation of this study, Pathak *et al*. (2020) reported the separation of all the developmental stages between a mutant and its respective wild type using principal component analysis of gene expression data. The clustering of the FNI mutants into different clustered groups could be attributed to its effects on the DNA of the treated plants depending on the intensity, leading to the appearance or disappearance of nucleotide bands in DNA-polymerase chain reaction analysis. Point, insertion and/or deletion mutations, as well as more severe forms of DNA damage that prevent subsequent replication of the genome, had earlier been reported to arise due to ionizing radiation (Chatterjee and Walker, 2017). Fadia *et al*. (2011) studied irradiated seeds of *Hibiscus sabdariffa* L. with gamma rays and reported the disappearance of DNA polymorphic bands in response to treatment doses.

Conclusion

The high percentage of polymorphism and gene diversity obtained among mutants and their parent (control) using RAPD primers indicate the effectiveness of the markers in studying the genetic diversity of mutants *Celosia argentea*. The clustering of the mutants and their parent into five distinct genetic groups by UPGMA dendrogram and 4 groups by principal component analysis confirmed the high level of induced polymorphism among the treatments. Therefore, Sodium Azide and Fast Neutron Irradiation are

effective mutagens for inducing useful variability in *Celosia argentea.*

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Table 1: Major allele frequency, percentage polymorphism, genetic diversity and polymorphism information contents on control and mutant plants of SA and FNI

Marker		Major Allele Frequency	Number of Monomorphic	Number of Polymorphic	(%) Percentage Polymorphism	Allele Number	Genetic Diversity	PIC
OPH05	5^1 - GGG TTT GGC A - 3^1	0.40	0.00	6.00	100	5.00	0.74	0.70
OPB17	5^1- AGG GAA CGA G - 3^1	0.70	0.00	6.00	100	4.00	0.48	0.45
OPT17	5^1 - CCA ACG TCG T - 3^1	0.40	0.00	4.00	100	5.00	0.72	0.68
OPB11	5^1 - GTA GAC CCG T - 3^1	0.50	0.00	2.00	100	2.00	0.50	0.38
OPB04	5^1 - GGA CTG GAG T - 3^1	0.40	0.00	3.00	100	3.00	0.64	0.56
Mean		0.48	0.00	4.00	100	3.80	0.62	0.55

Table 2: Dice Dissimilarity Index of control and mutant plants of SA and FNI

OUT	Parent	Buffer	2mM	4mM	6mM	8mM	30min	60min	90min	120min
Parent	0.00	1.00	0.60	1.00	0.80	1.00	0.60	0.60	1.00	0.40
Buffer		0.00	0.80	0.20	0.40	0.20	0.80	0.80	0.80	0.80
2 mM			0.00	0.80	0.80	0.80	0.60	0.20	1.00	0.60
4 mM				0.00	0.40	0.20	0.60	0.80	0.60	0.80
6 mM					0.00	0.40	1.00	0.80	0.80	0.60
8 mM						0.00	0.60	0.80	0.60	0.80
30min							0.00	0.60	0.80	0.80
60min								0.00	1.00	0.60
90min									0.00	0.80
120min										0.00

mM - Millimolar

Table 3: Principal Component for Mutants Lines of *Celosia argentea* and its Parent

Mutants	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
Parent	1.49	-0.17	0.88	0.60	-0.23	0.12	0.64	-0.01
Buffer (Control)	-1.33	-0.72	-0.28	-0.30	-0.42	0.41	0.02	0.30
2 mM	1.40	-0.33	-0.51	-1.24	0.05	0.39	-0.04	-0.19
4 mM	-1.33	-0.56	-0.14	0.09	-0.16	-0.32	0.04	-0.16
6 mM	-1.13	-0.04	0.94	0.01	0.93	0.23	-0.02	0.02
8 mM	-1.33	-0.56	-0.14	0.09	-0.16	-0.32	0.04	-0.16
30mins	0.59	0.28	-1.54	1.17	0.26	0.19	-0.08	0.00
60mins	1.30	-0.13	-0.28	-0.53	0.27	-0.69	0.03	0.22
90mins	-0.73	2.32	0.07	-0.34	-0.23	-0.02	0.05	0.00
120mins	1.06	-0.09	0.99	0.45	-0.32	0.00	-0.68	0.00
Eigen value	1.61	0.75	0.61	0.44	0.16	0.12	0.10	0.02
% variance	42.15	19.72	15.99	11.51	4.16	3.22	2.61	0.65
Cumulative %	42.15	61.87	77.85	89.36	93.52	96.74	99.35	100.00

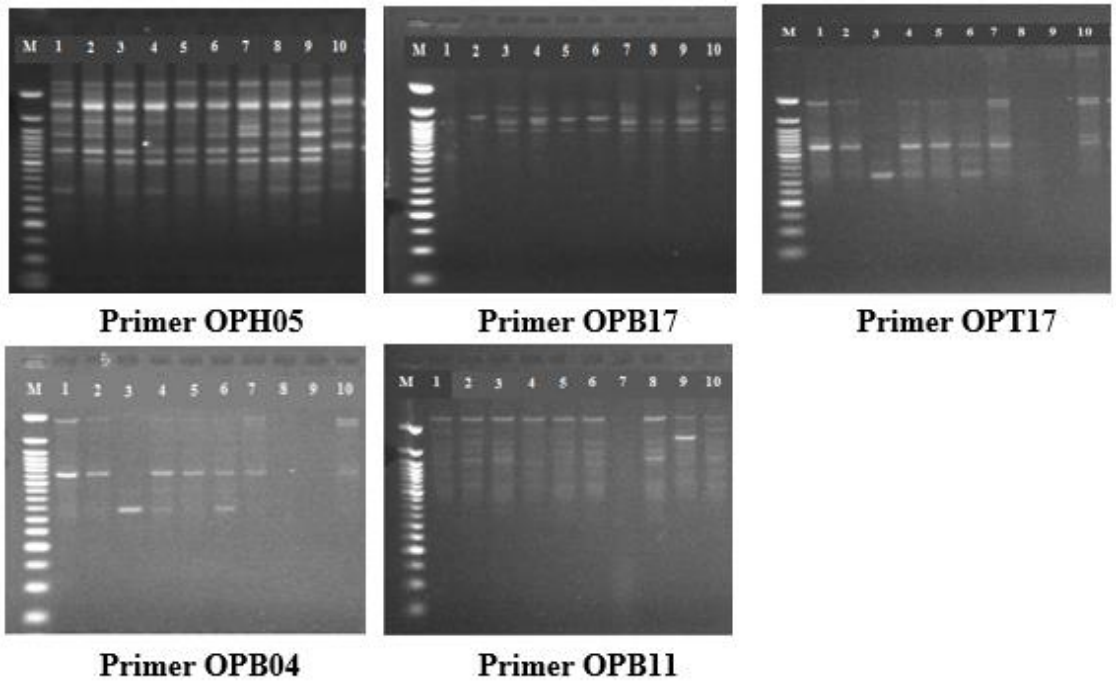

Figure 1: RAPD Amplification Patterns Using 5 Different DNA Primers
M = ladder and numbers 1 to 10 on the first row represent *Celosia argentea* parent and mutants.
1-parent; 2-Buffer; 3- 2mM; 4- 4mM; 5- 8mM, 6-30mins 7-60mins, 8-90mins & 10-120mins

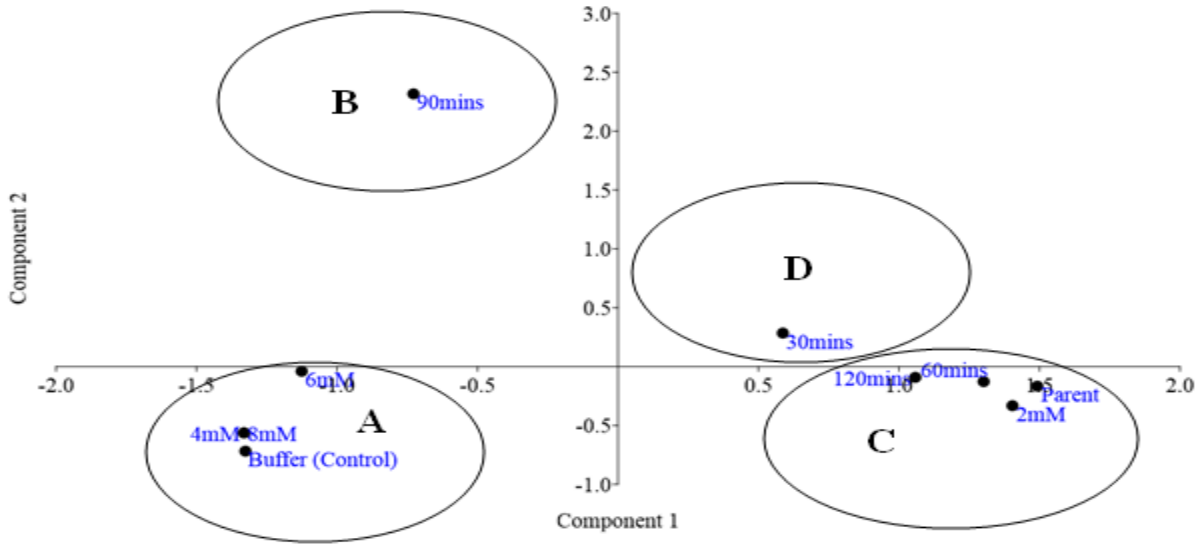

Figure 2: Scatter plot of Principal Component for Mutagenic Plants and its Parent

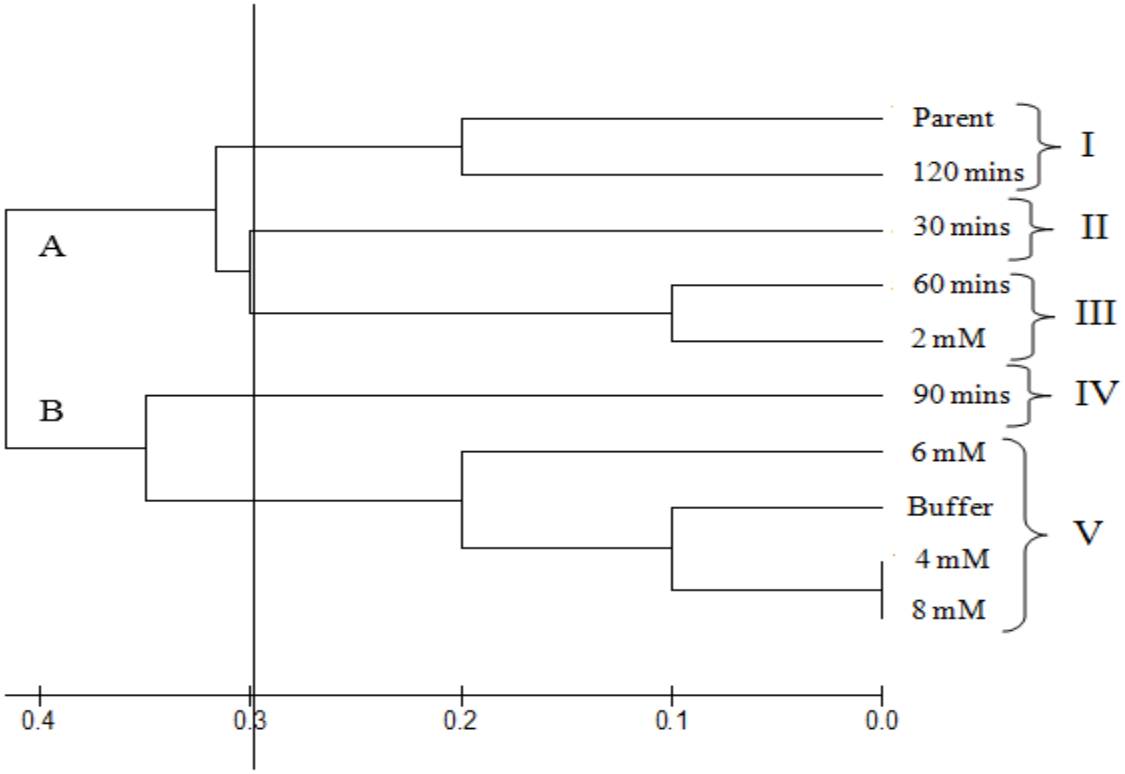

Figure 3: UPGMA dendrogram of mutant lines of *C. argentea* and its parent