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یافته‌های نوین در علوم زیستی
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القای تغییرات ژنتیکی و مورفولوژی در کاتارانتوس روزئوس توسط پلاسمای اتمسفری سرد

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چکیده. گیاه کاتارانتوس روزئوس به طور گسترده‌ای در جهان کشت می‌شود. پلاسمای سرد اتمسفری به منظور بهبود جوانه زنی بذر و رشد گیاهان زراعی استفاده می شود. در تحقیق حاضرسه زمان مختلف پلاسمای اتمسفری سرد(۴۰،۵۰و۶۰ ثانیه) به منظور تیمار بذرها استفاده و تغییرات در صفات ریختی، آنزیم‌های آنتی‌اکسیدانی و محتوای ژنتیکی در این گیاهان بررسی شد. پلاسمای سرد (۵۰ ثانیه) به طور قابل توجهی جوانه زنی، طول برگ‌ها و طول ساقه گیاهان را نسبت به گیاهان کنترل افزایش داد. فعالیت آنزیم های کاتالاز و پراکسیداز بالاترین میزان را در گیاهان تیمارشده با پلاسمای سرد به ترتیب در ۴۰ و ۶۰ ثانیه نشان دادند. نشانگر پلی مورفیسم تکثیریافته مرتبط با توالی (SRAP) بالاترین درجه تنوع ژنتیکی در گیاهان تیمارشده ۵۰ ثانیه پلاسمای سرد نشان داد (Ne = 1.388, I = 0.316, He = 0.217, uHe = 0.237 and P % = 50.08). بر اساس روش نزدیکترین خویشاوندی (NJ)، آنالیز مولفه های اصلی (PCoA) و آنالیز واریانس مولکولی (AMOVA)، چهار گروه مجزا شکل گرفت. گیاهان تیمار شده با ۴۰ و ۵۰ ثانیه پلاسمای سرد به علت تفاوت ژنتیکی با فاصله از گیاهان گروه کنترل قرار گرفتند. نتایج نشان می دهد پلاسمای اتمسفری سرد می‌تواند به عنوان ابزاری ایمن از لحاظ اقتصادی و زیست محیطی در افزایش رشد *C. roseus* و القای تغییرات ژنتیکی استفاده شود.

واژه های کلیدی. پروکسیداز، پلی مورفیسم تقویت شده مرتبط با توالی، تنوع ژنتیکی، کاتالاز، گل پریوش

Induced genetic and morphological changes in *Catharanthus roseus* L. by cold atmospheric plasma

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Abstract. *Catharanthus roseus* is widely cultivated around the world. Cold atmosphere plasma (CAP) has been used to improve crop plants seed germination, and plant growth. In the present study, three different times of CAP (40, 50 and 60s) were used for the treatment of *C. roseus* seeds, and the changes in morphological traits, antioxidant enzymes and the genetic content of the treated plants were examined. Cold plasma (50s) markedly raised the seed germination, length of the leaves and the stem length of the plants in comparison with those in the control group. The catalase and peroxidase enzyme activities had the highest value in 60 and 40 s CAP treated plants, respectively. The sequence related amplified polymorphism (SRAP) markers showed the highest degree of genetic diversity in 50s cold plasma treated plants (Ne = 1.388, I = 0.316, He = 0.217, uHe = 0.237 and P % = 50.08). Based on Neighbor Joining, principle coordination analysis (PCoA) and analysis of molecular variance (AMOVA) test, four significantly distant groups were formed. The 40s and 50s cold plasma treated plants stand far from the control plants due to genetic difference. The results indicate that cold atmosphere plasma could be used as an economic and environmentally safe tool in increasing *C. roseus* growth characteristics in addition to inducing genetic variations.

Keywords. catalase, genetic diversity, peroxidase, rose periwinkle, SRAP

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INTRODUCTION

Catharanthus roseus (L.) G.Don, known as periwinkle, belongs to Apocynaceae family, with high medicinal values is one of the most important ornament plants in the world. Among 6 species of this genus, *C. roseus* is widely cultivated around the world. This medicinal plant is the commercial source of monoterpenoid indole alkaloids (MIA) with pharmaceutical activities such as anti-tumor, anti-inflammatory and anti-microbial properties (Koul *et al.*, 2013). Genetic changes are among the possible ways to increase *C. roseus* medicinal components. Gamma radiation, UV irritation, somaclonal variation are common ways of increasing growth and medicinal components such as antioxidants and alkaloids (Swanberg & Dai, 2008; Zargar *et al.*, 2010; Verma *et al.*, 2012; Fatima *et al.*, 2015).

Cold atmospheric plasma (CAP) is non-thermal, ionized gas which possesses reactive and energetic particles such as positive and negative ions, electrons, metastables, atoms, free radicals and photons (Fridman *et al.*, 2005; Hoffman *et al.*, 2013). Cold plasma is as fast, economic and environmentally safe and used in different commercial applications such as medicine, food processing and agriculture. CAP has been used in agricultural research such as the increase of seed germination, the breaking of dormancy, plant growth and deactivation of plant pathogens (Ling *et al.*, 2014; Sivachandiran & Khacef, 2017). In addition, CAP could activate anti-oxidant enzymes such as catalase, peroxidase and superoxidase (Jiang *et al.*, 2014).

Studying the genetic diversity in medicinal and crop plants is one of the important concerns for researchers. They use different molecular markers for this task. Sequence-related amplified polymorphism (SRAP) markers are dominant with primers targeting GC-rich exons and AT-rich promoters, introns, and spacers (Li & Quiros, 2001). Robarts & Wolfe (2014) reviewed literatures for the application of SRAP in plant biology. They reported that SRAPs might be considered suitable molecular tools for the study of diversity and genetic changes. The effect of cold plasma on DNA sequences of treated seeds is still unclear. Zhang *et al.* (2016) studied the effect of argon plasma on soybean sprouts via demethylation levels of genes, which were related to the energy metabolic pathway. Their investigation showed increasing demethylation levels of the sequenced region of ATP a1, a target of rapamycin (TOR), growth-regulating factor (GRF) 1–6, down-regulating ATP b1, TOR, GRF 5, and GRF 6 of 6-day-old soybean sprouts.

The objectives of the current study included the investigation of the influence of cold atmospheric plasma treatment on morphological and catalase and peroxidase enzyme activities as well as the effect of cold plasma on genetic changes on the basis of SRAP molecular markers.

MATERIALS AND METHODS

Plant material and Cold plasma treatment

C. roseus (Goldsmith) seeds were provided by Syngenta flowers (Netherland). Four 48-well plates containing at least 100 wet seeds were used as control, 40, 50 and 60 seconds cold atmosphere plasma treatments. Cold atmosphere plasma jet provided by a Pyrex nozzle tube (inside diameter (ID) = 2 mm and outside diameter (OD) = 4 mm). The inlet gas for this study was helium (99.99%). the flow rate of He was 2 I/min. The power supply produced a 10 kV high voltage pulsing with a frequency of 6 kHz and 30 µs pulse width.

The seeds were sown in plastic pots (volume 500 mL), filled with fin pitte and perlite (3:1) soil. The pots were maintained in a growth chamber with 25/15 °C and 16/8h photoperiod. Plants with 5-6 leaves were transferred into bigger pots (volume 1000 ml, Fig. 1).

The morphological characters including the number of leaves and stem length were measured for each group using digimizer software. Characters were measured from the third week after seed cultivation till the seventh week.

Assays of antioxidant enzyme activities

Catalase and peroxidase enzymes were extracted using 1 ml extraction buffer (1.2gr Tris, 2gr ascorbic acid, 3.8 borax, 2gr EDTA and 50 gr PEG) for one gram of the homogenized fresh leaves. The homogenate solution kept at 4 °C for 24 hours, samples were then centrifuged for 15 min at 15000×g. The supernatant was used for enzyme activity assay (Ebermann & Stich, 1982).

The samples were prepared as described by Mukherjee & Choudhuri (1983). A leaf sample (0.5 g) was frozen in liquid nitrogen and ground using a porcelain mortar and pestle. The frozen powder was added to 10 ml of 100 mM phosphate buffer (KH2PO4/K2HPO4) pH 7.0, containing 0.1 mM Na2EDTA and 0.1 g of polyvinylpyrrolidone. The homogenate was centrifuged at x 15,000 g for 10 min at 4°C., and the resulted supernatant was collected and stored at 4°C for CAT, POD, APX and SOD assays (Azooz *et al*., 2012).

Catalase (EC 1. 11. 1. 6) activity was assayed according to Aebi (1984).

The activity of catalase was estimated by the decrease of absorbance at 240 nm for 1 min as a consequence of H2O2 consumption as described by

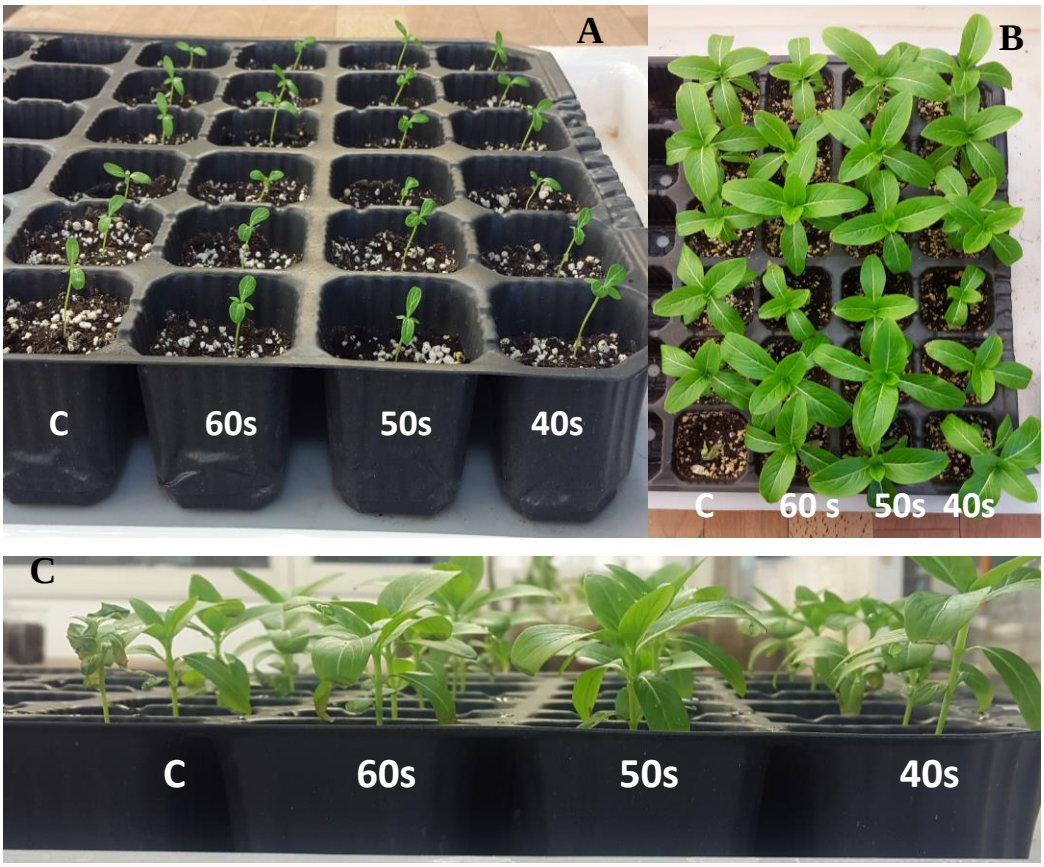


Fig.1. Plant growth in the four treatments of 3 (A), 5 (B) and 7 (C) weeks. C= control, 40s, 50s and 60s = time of cold plasma treatments

Havir & Mellate (1987). Peroxidase (EC 1. 11. 1. 7) activity was determined according to Maehly & Chance (1954) by the oxidation of guaiacol in the presence of H2O2. The increase in absorbance due to the formation of tetraguaiacol was recorded at 470 nm.

SRAP markers analysis

Fresh leaves of 5 plants of each treatment were randomly collected for DNA extraction. Genomic DNA extraction was carried out on the basis of the CTAB method with modification (Krizman *et al*. 2006). The Quantity and quality of the extracted DNA were evaluated by nanodraop spectrophotometer and 0.8% agarose gel electrophoresis. For genetic variation analysis, 15 different combinations of forward and reverse SRAP primers were used for PCR amplification (Table 1).

The amplification reaction was carried out in a Bio Rad thermal cycler with a touchdown PCR program: 94 ºC for 5 min, followed by 5 cycles of 94 C for 1 min, 35 C for 45 sec, and 72 C for 1 min, and 35 cycles of 94 C for 1 min, 50 C for 45 sec, 72 C for 1 min, and a final extension at 72 C for 5 min. PCR mixture contained 30 ng template DNA, 2.0 µl of 10X PCR buffer, 1.5 µl of 50mM MgCl2, 1.0 µl of 10mM dNTPs, 0.6 µl of 10mM each of forward and reverse primers, and 0.5 µl of 5 unit/µl Taq DNA polymerase. PCR products were separated on 2.5% agarose gel and visualized by GelRed stain. The band patterns obtained by each pair of SRAP primers were scored as present (1) or absent (0).

Data analyses

Morphological characters were measured in all plants of 4 treatments. ANOVA (Analysis of variance), followed by LSD test used to study significant difference among the samples.

Genetic diversity parameters were determined in each of the treatments studied. The expected heterozigosity (He), Shannon information index (I), number of effective alleles, and the percentage of polymorphism were analyzed by GenAlex ver 6.5 (Weising *et al.*, 2005; Freeland *et al.*, 2011). For differentiation of the groups studied, principal coordinate analysis (PCoA) was performed after 1000 times bootstrapping/ permutations (Freeland *et al.*, 2011).

RESULTS

In the present study, the leaf and stem lengths were measured for each of the groups in 5 consequent weeks (third week till seventh week). The results showed no significant ($P > 0.050$) differences between leaf and stem lengths of control and cold plasma treated plants (Table 2). However,

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Table. 1. SRAP primer combinations and their sequences (Feng *et al.*, 2014; Abedian *et al.*, 2012).

Combination	5′ – 3′ Sequences
Me 1_ Em 2	TGAGTCCAAACCGGATA _ GACTGCGTACGAATTTGC
Me 1_ Em 3	TGAGTCCAAACCGGATA _ GACTGCGTACGAATTGAC
Me 1_ Em 4	TGAGTCCAAACCGGATA _ GACTGCGTACGAATTTGA
Me 2_ Em 1	TGAGTCCAAACCGGAGC _ GACTGCGTACGAATTAAT
Me 2_ Em 2	TGAGTCCAAACCGGAGC _ GACTGCGTACGAATTTGC
Me 2_ Em 3	TGAGTCCAAACCGGAGC _ GACTGCGTACGAATTGAC
Me 3_ Em 1	TGAGTCCAAACCGGAAT _ GACTGCGTACGAATTAAT
Me 3_ Em 2	TGAGTCCAAACCGGAAT _ GACTGCGTACGAATTTGC
Me 3_ Em 4	TGAGTCCAAACCGGAAT _ GACTGCGTACGAATTTGA
Me 4_ Em 2	TGAGTCCAAACCGGACC _ GACTGCGTACGAATTTGC
Me 4_ Em 3	TGAGTCCAAACCGGACC _ GACTGCGTACGAATTGAC
Me 4_ Em 5	TGAGTCCAAACCGGACC _ GACTGCGTACGAATTAAC
Me 5_ Em 1	TGAGTCCAAACCGGAAG _ GACTGCGTACGAATTAAT
Me 5_ Em 3	TGAGTCCAAACCGGAAG _ GACTGCGTACGAATTGAC
Me 5_ Em 4	TGAGTCCAAACCGGAAG _ GACTGCGTACGAATTTGA

in 40s and 50 s cold plasma treatments length of leaves and stem length were increased in comparison with control plants (Table 2). The measurement of germination rate showed the highest amount in 50 s cold plasma treated seeds.

Catalase and peroxidase enzyme activities were measured in three treated and the control plants. The 60 s cold plasma treated plants showed highest, while the lowest amount of catalase activity belonged to the control plants. On the other hand, peroxidase activity showed the highest value in 40 s cold plasma treated plants. However, all treated plant groups showed significant differences with control plants in both enzymes studied (Table 3).

SRAP Analysis

In regards to the analysis of genetic variation between cold plasma treated and control plants, 15 SRAP markers were used. Banding pattern of one of the SRAP primer pairs (Em2-Me1) is shown in Fig. 2. Based on 23 samples in 4 groups, SRAP markers had mean values of the number of effective allele (Ne = 1.318), Shannon index (I = 0.262), Nei genetic diversity (He =0.179), unbiased expected heterozygosity (UHe = 0.196) and the percentage of polymorphism (P% = 46.17, Table 4). Among the treated groups, 50s cold plasma treated group showed the highest diversity (Ne = 1.388, I = 0.316, He = 0.217, uHe = 0.237 and P% = 50.08), while, 40s cold plasma treated samples showed the lowest genetic parameters (Ne = 1.281, I = 0.233, He = 0.158, uHe = 0.173 and P% = 41.84).

For differentiation analysis, Neighbor Joining (NJ, Fig. 3) and principle coordinate analysis (PCoA) were performed on SRAP data. Based on NJ cluster, four distant groups were formed. Meanwhile, 40s and 50s cold plasma treated plants stood far from controls, showed genetic differences between them. AMOVA test also showed significant differences between these four groups (PhiPT=0.281, P value =0.001).

The PCoA plot (Fig 4) showed that the control plants were distinctly separated from 50s cold plasma treated plants. The same was true for 40s and 60s treated plants (Fig.4 A, C). The highest genetic distance was detected between control and 50s cold plasma treated plants (D = 0.184) based on Nei genetic distance analysis (data not shown). The PCoA analysis also supported this result.

DISCUSSION

C. roseus is one of the most important medicinal plants. Recently, cold plasma as an eco-agricultural technology, plays a main role in developmental and physiological processes in plants. However, no information is available on the effect of cold plasma

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Table 2. Descriptive results of morphological traits and ANOVA test in 4 groups (control, 40s, 50s and 60s cold plasma treatments) and 5 consequent weeks.

Treatments	Third week		Fourth week		Fifth week		Sixth week		Seventh week	
	Length of leaves (cm)	Length of stems (cm)	Length of leaves (cm)	Length of stems (cm)	Length of leaves (cm)	Length of stems (cm)	Length of leaves (cm)	Length of stems (cm)	Length of leaves (cm)	Length of stems (cm)
Control	2.00 ± .00	2.817 ± .1887	4.00 ± .00	3.267 ± .1498	6.00 ± .00	3.933 ± .2716	7.60 ± .40	5.960 ± .4179	10.60 ± .748	7.020 ± .3541
40s	2.00 ± .00	2.683 ± .2104	4.67 ± .422	3.583 ± .3516	6.67 ± .667	4.317 ± .3135	8.33 ± .955	6.050 ± .5926	11.83 ± 1.558	6.733 ± .6601
50s	1.83 ± .167	2.800 ± .2295	3.83 ± .167	3.467 ± .2248	5.83 ± .167	4.500 ± .2066	8.33 ± .615	6.750 ± .2952	11.00 ± .447	7.517 ± .4045
60s	2.00 ± .00	2.733 ± .1145	4.00 ± .00	3.200 ± .10	6.00 ± .00	3.900 ± .1826	6.67 ± .422	5.917 ± .240	10.83 ± .833	6.800 ± .3022
P value	0.413	0.957	0.076	0.618	0.351	0.274	0.245	0.444	0.837	0.612

Table 3. Catalase and peroxidase enzyme activities in 4 groups studied. SE: standard error.

Treatment	Catalase	peroxidase
Control	0.02352 SE= .005	0.0095 SE= 0.0046
Plasma (40s)	0.03057 SE= 0.005	0.0129 SE= 0.0035
Plasma (50s)	0.00346 SE= 0.001	0.0077 SE= 0.0012
Plasma (60s)	0.05273 SE= 0.005	0.0092 SE= 0.0043

Table 4. Genetic parameters based on SRAP data in 4 groups studied

Group		Ne	I	He	uHe	P%
Control	Mean	1.307	0.256	0.173	0.192	46.94
	SE	0.040	0.030	0.021	0.023	
40s cold plasma	Mean	1.281	0.233	0.158	0.173	41.84
	SE	0.039	0.030	0.021	0.023	
50s cold plasma	Mean	1.388	0.316	0.217	0.237	54.08
	SE	0.041	0.031	0.022	0.024	
60s cold plasma	Mean	1.298	0.244	0.167	0.182	41.84
	SE	0.040	0.030	0.021	0.023	
Total	Mean	1.318	0.262	0.179	0.196	46.17
	SE	0.020	0.015	0.011	0.012	2.90

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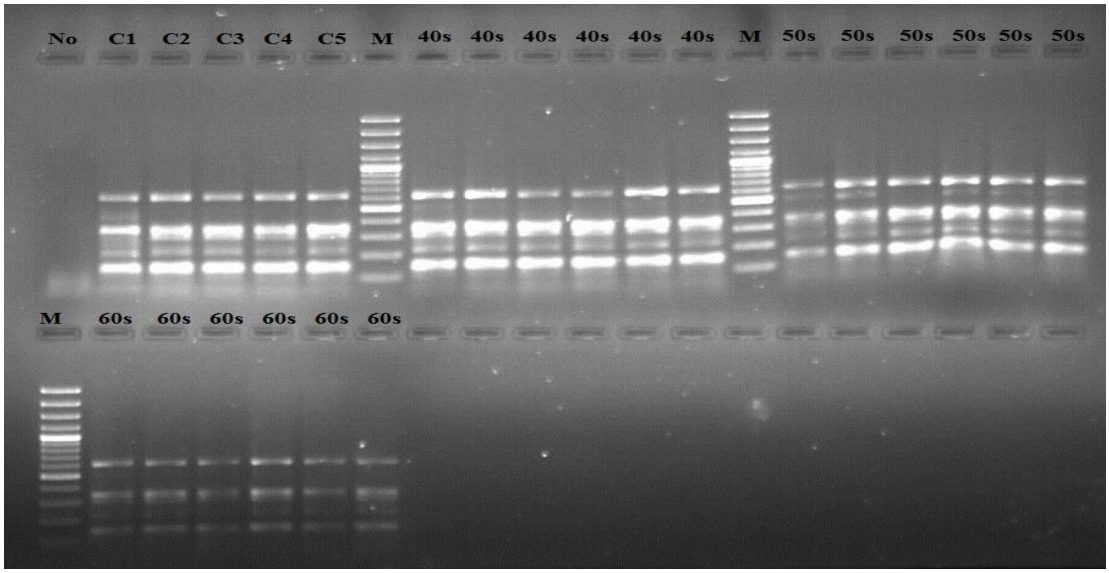


Fig. 2. Banding pattern of Em2-me1primer pairs on 4 groups studied.

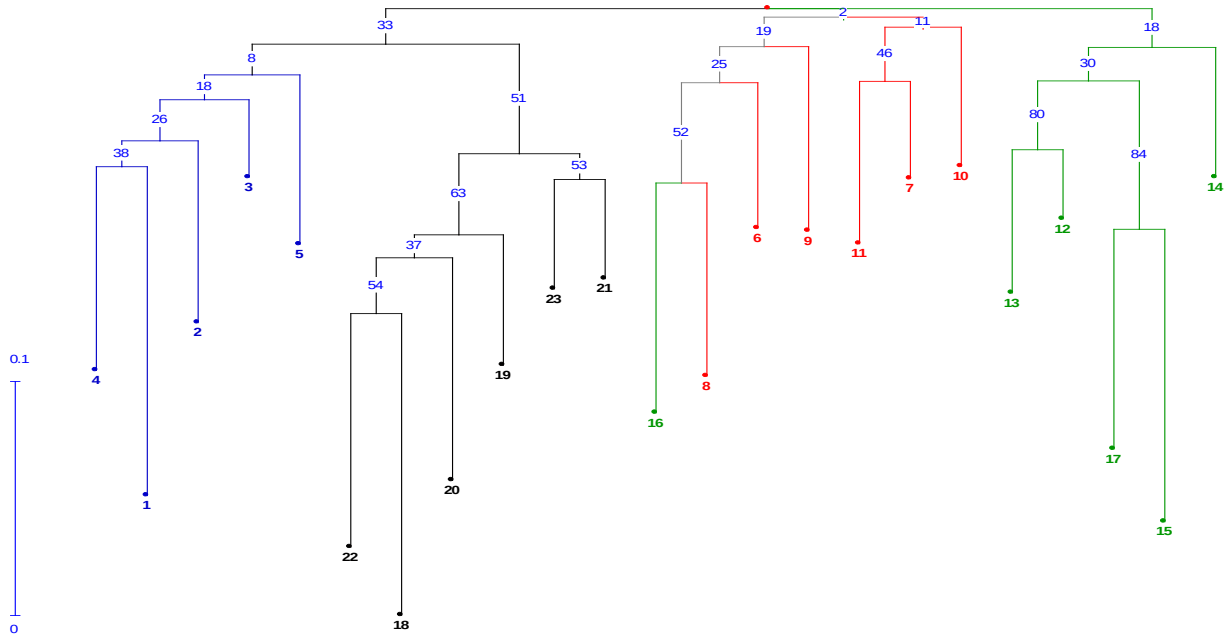


Fig. 3. Neighbor Joining clustering on control and treated samples based on SRAP data. Blue lines = controls, Red line = 40s cold plasma treated samples, green line = 50s cold plasma treated samples and black line = 60s cold plasma treated samples. Numbers on line, bootstrapping in 1000 permutation.

on genetic changes, especially on *C. roseus*. In the present study, genetic variations have been investigated in three different cold plasma exposure times using SRAP markers. Plant growth characteristics, namely length of leaves and stem were measured in control and cold plasma treated plants. Although no significant differences were observed in plant growth, considerable increases were detected in the growth of leaves and stems in the treated plants. The stimulating effect of cold plasma on the seed germination and seedling growth have been reported in different plants like tomato, sweet potato, wheat, oat, peanut and maize in different cold plasma exposure times (Sivachandiran & Khacef, 2017; Meng *et al*., 2017; Šerá *et al*., 2010; Meiqiang *et al*., 2005; Ling *et al*., 2016; Jianfeng *et al*., 2014; Henselová *et al*., 2012).

However in some reports, longer exposure-time related positively to the decrease of root or stem growths (Sivachandiran & Khacef, 2017; Meng *et al*., 2017; Henselová *et al*., 2012). Cold plasma affects the characteristics of seeds and can increase the hydrophilic ability of seed to improve water uptake. Wettability along with increasing in nutrition absorption promotes plant growth (Alves Junior *et al*., 2016). Souza *et al.* (2014) reported that

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the increase in leaf area in cold plasma-treated plants caused increasing photosynthetic rate.

Antioxidant enzymes are responsible for the accumulation and detoxification of hydrogen peroxide and reduce deleterious effects of H2O2 in stress responses (Caverzan *et al.*, 2012). Catalase and peroxidase enzymes play this role in plants. Yin *et al.* (2005) reported that cold plasma treatment on tomato seeds increased catalase, superoxidase and peroxidase enzyme activities and consequently improved ATP and metabolism of active oxygen.
The same results have been reported by Zhang *et al.* (2017) in soybean sprouts seeds by argon plasma pre-treatment. In the present study, catalase enzyme also increased in cold plasma treated plants at different exposure times. The highest catalase activity was observed in 60 second cold plasma treated plants at different exposure times. The highest catalase activity was observed in 60 second cold plasma-treated plants while the highest peroxidase activity was in 40 second cold plasma-treated plants. The catalase activity is highly associated with the ability of plants to tolerate different stresses. One mechanism for active oxygen scavenging is increasing enzymes such as catalase, peroxidase and superoxide dismutase. Cold plasma treatment transfers exogenous H2 O2 to seeds may dramatically increase the oxygen scavenging ability of treated plants (Apel & Hirt, 2004; Li & Xue, 2010; Zhou *et al.*, 2016). In the present study, genetic variation in control and cold plasma-treated plants were examined by SRAP markers. Due to the nature of SRAP markers, they target coding, regulatory and intron sequences.

The effect of cold plasma on plant genomes is still unclear. It is believed that stress response in plants may be caused by the variation in methylation pattern in regulatory sites (Uthup *et al.*, 2011; Chan *et al.*, 2005; Lippman *et al.*, 2004). Zhang *et al.* (2017) reported the increase of demethylation level of sequences in *ATP a1, ATP b1, TOR, GRF 5,* and *GRF 6* of soybean sprouts treated by plasma at

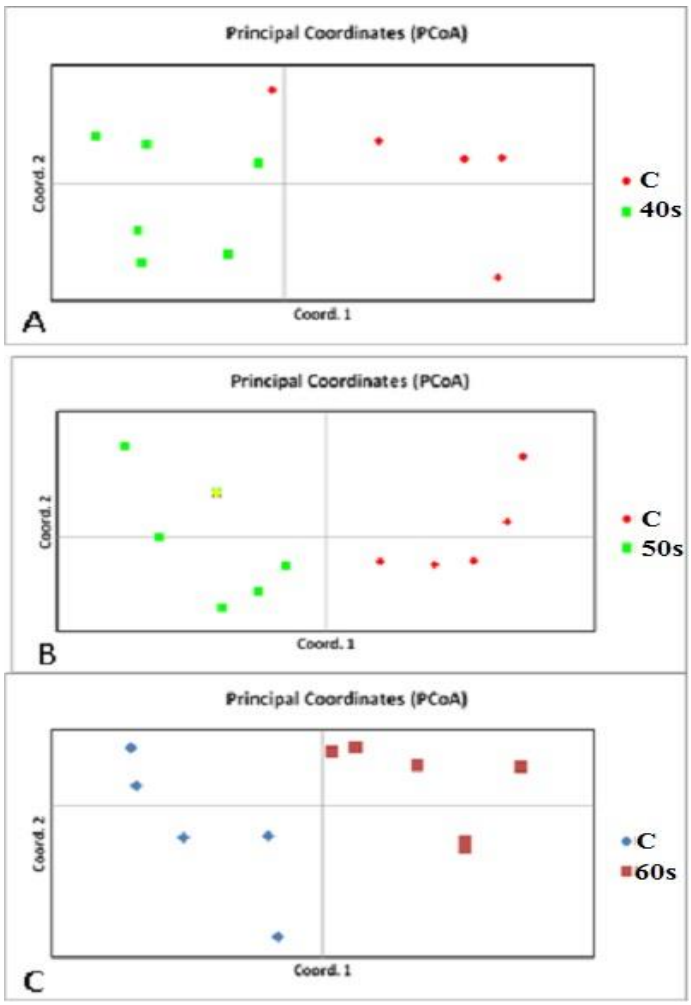


Fig. 4. PCoA ordinations based on SRAP data. **A:** ordination between control and 40s cold plasma treated plants, **B:** ordination between control and 50s cold plasma treated plants, and **C:** ordination between control and 60s cold plasma treated plants.

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22.1 kV for 12 s. The methylation of regulatory sequences is related to changes in gene transcription, while demethylation of CpG islands is associated with transcriptional activation of selected imprinted genes (Maeder *et al*., 2013).

SRAP markers, due to the nature of their primers (CCGG in the forward primer and AATT sequences in the reverse primer), cover coding sequences, as well as promoter regions. These markers have been proved to be powerful for agronomic purposes (Robarts & Wolfe, 2014). In the present study, 40s and 50 cold plasma-treated plants showed genetic distances from control plants. It may come from genetic changes in DNA sequences which SRAP markers have been targeted.

In conclusion, the cold plasma jet could change seed germination, morphological traits as well as antioxidant enzymes. In addition it could create genetic variations in genome of *C. roseus*. We also estimated the proper time of cold plasma-treatment. The cold atmosphere plasma could be useful as economically and environmentally safe tools for the increase of *C. roseus* growth and genetic variations.

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