Biosaintifika 8 (3) (2016) 344-351

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

In Vitro Selection of Local Maize (*Zea mays*) on NaCl Stress and its Genetic Characterization using RAPD

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DOI: 10.15294/biosaintifika.v8i3.6934

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History Article

Received 20 August 2016
Approved 5 October 2016
Published 24 December 2016

Keywords:

Callus; Selection; NaCl; Random Amplified Polymorphism DNA (RAPD)

Abstract

Maize (*Zea mays*) is one of graminae plants that widely spread for many purposes wheter in food industry, feed, or bioenergy. Those high utilization required an increment in production, but unfortunately the the demands not in accordance with the volume of production since conversion of agricultural area increase lately. Indonesia has many of shoreline that recognized as marginal land where the salinity is high as well. This research try to obtain tolerant variant from two local cultivars that planted in Madura Island. Manding and Talango varieties were used as an explant for callus induction stage in MS supplemented with 2,4 D. The result showed that 4 ppm of 2,4 D were the best concentration to induce the callus in both varieties. The induced callus were exposed to medium MS that contained NaCl (0, 2500, 5000, and 7500 ppm). In 7500 ppm of NaCl, Manding variety has 100% of surviving callus, while Talango variety only 66,7%. Furthermore, Manding variety showed a better performance in callus weight improvement with 170 mg, while Talango gave no improvement in callus weight. The result of RAPD analysis indicated that the genome characteristic was different between initial callus and surviving callus. Only five primers were presence polymorphism i.e OPA 13, OPB 07, OPC 02, OPK 20, and OPU 19 from ten in total primers. Manding elected as high tolerance variety in Salinity stress, thus it proposed to be developed furthermore.

How to Cite

Saputro, T. B., Finariyah, F., Dianawati, S., Sholihah, N. F., & Ermavitalini, D. (2016). *In Vitro* Selection of Local Maize (*Zea mays*) on NaCl Stress and its Genetic Characterization using RAPD. *Biosaintifika: Journal of Biology & Biology Education*, 8(3), 344-351.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Maize (*Zea mays*) is one of perspective species in world plants utilization. It was ranked in top three cereal for the most planted crop beside wheat and rice worldwide. In addition it has high worldwide productivity as human food and fodder as well as source of large number of industrial products. Recently, the demands increased higher than total maize production. Land conversion into industrial area or building contributed for the decrement of maize production. Furthermore, coastal zone belong to the marginal land which promising to extend the plantation area of maize. Coastal zone is widely spread in Indonesia with high content of salt. High salinity content was remained as a challenge for maize production since maize is very sensitive toward high salinity level.

Salinity defined as a condition of soil that had an excessive concentration of dissolved salt (Yuniati, 2004). Salinity stress was presumably as environmental stress that causing a significant reduction in plant production (Mahajan and Tuteja, 2005). It was a major trouble for both plant growth and productivity (Flowers, 2004). The difficulties to find tolerant genes over salinity stress encourage the scientist to induce the natural variants through somaclonal variation or mutation induction in term to improve the variation among varieties. In this research, we try to combine somaclonal variation and in vitro selection to improve the local variety against salinity. Somaclonal variation and in vitro mutagenesis combination were beneficial in salinity isolation and drought tolerant regarding short duration within in vitro selection (Samad *et al* 2001). *In vitro* selection selected salt tolerance (Rosas *et al* 2003) and shortens the time considerably for desirable trait under selection pressure in maize (Balkhrisna and Shankarro, 2013). Evaluation and characterization of the spontaneous and induced variants against salinity proved highly fruitful venture for its successful cultivation in stress conditions. Moreover, various molecular techniques such as RFLP, AFLP, RAPD, SSR, SNP were frequently used to characterize the induced genetic variation. Among these molecular techniques, The characterization using Random Amplification Polymorphic DNA (RAPD) was simple, quick, easy to perform, required small amount of DNA for analysis and the major advantage was no prior sequence information required (Williams et al 1990).

RAPD was the first molecular marker technique that developed based on Polymerization Chain Reaction (PCR). In RAPD technique, the PCR primer has only 10 bases (decamers), this condition allowed random amplification for suitable segment of DNA (Williams et al., 1990). RAPD widely used to detect variation and characterize the identity of plant. Correa in 1999 used RAPD to see genetic distance in Soy bean. The DNA band that produced at RAPD technique was very consistent for many primers and this technique had played on various plants such as rice, corn, coffee and in orchid plants (Hoon-Lim *et al*. 1999). Molecular characterization using RAPD analysis revealed genetic polymorphism between the selected salt and drought tolerant lines from the control plants (Patade *et al*, 2006). This report described an effective and rapid method to improve the variant of maize by combined the induction of callus, in vitro selection and genetic variety characterization by RAPD technique and also compared between Talango and Manding in respond the stress.

METHODS

The research was conducted in the laboratory of Botany laboratory of Biology department of Institut Teknologi Sepuluh Nopember Surabaya in april 2014 to december 2014. Talango and Manding varieties carried out from Madura island, East Java as the explant, medium composed as basal medium Murashige and Skoog (MS), 2, 4-Dichlorophenoxy Acetic Acid (2.4 D), sodium chloride (NaCl), an isolation DNA reagent, 10 random decamer primers IDTDNA, PCR reagents KAPA and electrophoresis reagents.

The kernels had taken from mature and viable seeds of Talango and Manding varieties. It was a collection of Botany laboratory of Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia. Approximately 3 grams of mature seeds were surface sterilized with detergent in 5 minutes, and then rinsed in tap water. It was carried out into the laminar air flow cabinet for the next sterilization steps. It followed by soaked into 70% ethanol for 2 min and rinsed with distilled water. It treated with 1.5 % of NaOCl (Sodium hypochloride) for 3 minutes. To remove the surfactants, sterilized seeds were washed 3 times using sterile distilled water and blotted on to a sterile filter paper. The materials were ready to inoculate into medium after most of water were absorbed into the filter paper.

MS medium were used to induce the formation of callus. The MS supplemented with a various of 2,4-D concentration (0; 1; 2; 3; 4; and 5 ppm) to find an optimum number of callus formation and were incubated in the dark condition

at 25° C. After 4 weeks, the optimum concentrations were evaluated. In this research, we found that 4 ppm of 2,4-D induced the highest number of callus. They were subcultured into the selection medium that contained several concentration of NaCl 0 ppm (control), 1000 ppm , 2500 ppm , 5000 ppm , and 7500 ppm and incubated 27-28°C for 6 weeks.

After the selection process, genomic DNA isolated from the surviving callus. 80-100 mg of a callus isolated by using Cetyl Trimethyl Ammonium Bromide (CTAB) 3% technique. Isolated DNA then used as a template in PCR reaction and added one of each 10 different RAPD primers. PCR mixture had performed by KAPA reaction mix. PCR reaction programmed to 30 cycles as follows: First Denaturation 94°C for 5 minutes, 94°C for 1 minute, annealing 36°C for 1 minute, extension 72°C for 1 minute and the final extension 72°C. PCR product visualized in 2 % agarose gel.

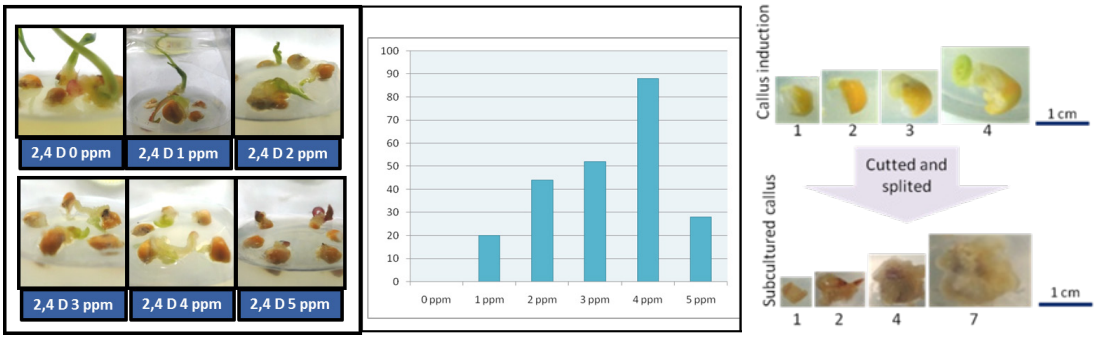
RESULT AND DISCUSSION

Every species had a different response to any addition of growth promoting substances on culture media. Pathi *et al* in 2013 reported that the maximum frequency of embryogenic callus formation (90%) was obtained on MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BAP in the dark conditions, while the compact granular organogenic callus formation (85% frequency) was obtained on MS medium supplemented with 2.5 mg/l 2,4-D and 1.5 mg/l BAP at light conditions. On the other hand, Gorji *et al*., 2011 using N6 medium supplemented with 2 mg/L Dicamba induced the highest frequency of organogenic callus. But, the usage of 2,4 D can make the selected callus easy to regenerated. Huang and Wei (2004) mentioned the role of 2,4-D with MS media in inducing highly regenerable calli from mature embryos.

Figure 1(A) showed that explants in control treatment were directly grow as a plantlet. Along with the increment of 2,4 D, the frequency of callus formed were also increase. On the other hand, 5 ppm of 2,4 D reduced the rate of callus form Figure 1(B). The optimum concentration that induced the highest amount (88%) of callus was 4 ppm of 2,4-D. Furthermore, 5 ppm of 2,4 D concentration produce the lowest percentage of callus formed, a small callus and also induced browning condition.

Solangi *et al* in 2015 reported that 2, 4-D at 3.0 mg/L concentration was the most effective auxin for callus proliferation and weight in all the sugarcane varieties tested. The optimum concentration of 2,4D condition were also well observed by Al-Abed *et al*. (2006) whereby the increase of 2,4-D level will decrease the induction of maize callus and resulted of browning of calli at ≥4 mg/L of 2,4-D. Pathi *et al* in 2013 mentioned that the rate of callus formation showed a decrement at lower concentration, while in higher concentrations 2,4-D and BAP negatively affected the callus formation rate. The development of callus formation for every week were well documented in Figure 1(C), all the calli formed were subcultured into the same medium, MS supplemented with 4 ppm 2,4-D for the first induction into subcultured were used the same medium.

The induction of callus in this research were conducted to increase the genetic variation in maize. The repetitive mitotic condition in cells causing a new varians called somaclonal variation. Somaclonal variation occurs among the tissue culture regenerated plants. Irregular mitotic process leading to chromosomal instability, occurrence of gene amplification or deletion, gene inactivation or reactivation of silent genes, transposition and somatic crossing over, DNA methylation and point mutations (Larkin and



A B C

Figure 1. (A) Callus induction on Talango variety with the addition of various 2,4 D concentration; (B) Graph of callus formation frequency on various 2,4 D concentration; (C) Development of callus during first induction and subcultured.

Scowcroft 1981; Muler *et al* 1990). Those condition induce the re-arrangement of chromosome during exposure time. But the selection should be conducted for several times to gain the stability of those re-arrangement.

Genetic variation is the main source for plant breeding and biotechnology in term to provide various of germplasm (Anwar *et al*, 2010). Somaclonal variations of genetic nature might arise from alterations of chromosome or DNA sequence changes (Balkrishna and Shankarrao, 2013). Plant cells grown in vitro have been shown to be susceptible to genomic variations, a phenomenon often referred to as somaclonal variation. The frequency of variation depend on the genotype, culture medium, growth hormones and the multiplication technique (Khoddamzadeh *et al*, 2010). During the induction and maintenance phase of the callus is the most important time to induce the somaclonal variation, due to the larger period of time required by this phase compared to plant regeneration step and to the presence of a phytohormone (Vasconcelos *et al.*, 2008). A wide range of significant variation was observed for all the parameters in regenerated plants compared to control plants of potato (Anwar *et al*, 2010).

An induced callus had further propagated then subcultured on medium that contains a various concentration of NaCl i.e 0 ppm (control) , 2500 ppm, 5000 ppm, and 7500 ppm. In this stage several parameters were observed including callus morphology, percentage of surviving callus, and weight of callus.

Callus morphology in medium contains NaCl

Callus morphology exposed to NaCl for 28 days can be seen in Figure 2. The observation shows changes in color and texture of the callus exposed to NaCl. Callus experienced a browning and alteration of texture into a compact form. These changes occur in the medium with the highest concentrations of NaCl, while the callus without NaCl has a green color.

Callus exposed to NaCl shows a browning performance, Munir and Aftab (2013) reported the same result in sugarcane callus culture which is under salinity become browning and necrosis at the highest salinity concentration. The mechanism is caused by a decrease in chlorophyll content triggered by higher activity of Chlorophillase enzyme that degrade chlorophyll.

According to Sevengor *et al.*, (2011), chlorophyll degradation can be protected by the antioxidant enzyme activity. The activity is one form of defense mechanism against salinity stress (Kusvuran *et al.*, 2012). The black color is visible on the callus (Fig. 3) in Talango variety indicates the occurrence of death cell. This condition caused by the ionization of NaCl molecules into Na^+ and Cl^-, causing ion stress and lead to cell death of callus (Farid *et al.*, 2006). In addition, this browning-black condition are the result of phenolic compounds which are toxic compound for cells. This browning tissue were resulted by the activity of copper-containing oxidase enzymes such as polyphenol oxidase and tyrosinase (Hutami, 2008). Browning can also produced by endophytic microorganisms that are found in the tissues, whether no clear symptoms caused by bacteria or yeast (Prittila *et al.*, 2008).

High concentrations of salts has a huge effect on soil condition. Plant need to cope all those effect properly. At the cellular level, the Salt Overly Sensitive (SOS) signaling pathway

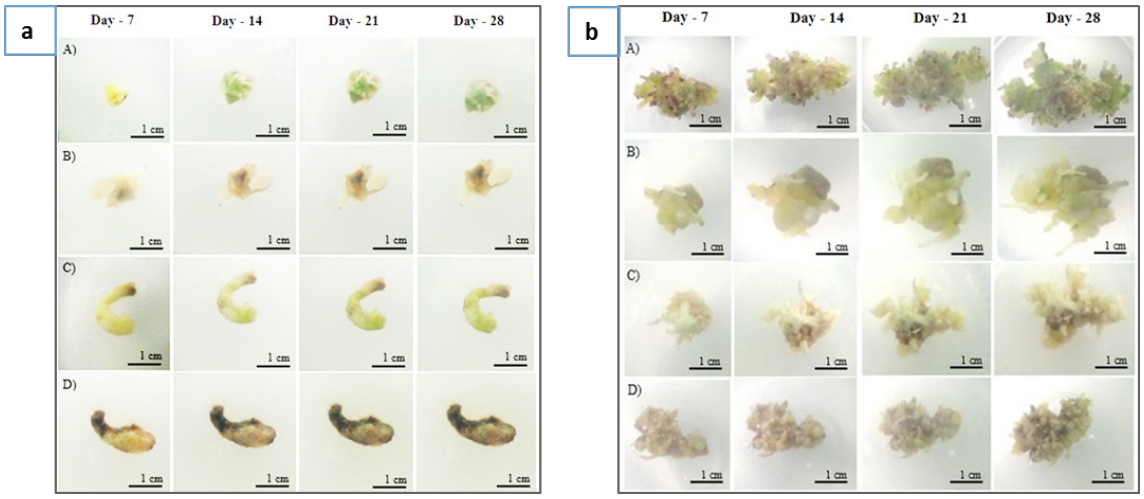


Figure 2. (A) Callus morphology of Talango variety exposed NaCl; (B) Callus morphology of Manding variety exposed NaCl. Note : A. 0 ppm, B. 2500 ppm, C. 5000 ppm, D.7500 ppm

that comprises SOS3, SOS2, and SOS1 has been proposed to mediate cellular signaling under salt stress, to maintain ion homeostasis (Ji *et al*., 2013). Dehydration-responsive element binding protein (DREB) is particularly to DRE elements and induces the stress tolerance gene expression. Over expression of genes encoding these proteins can induce high gene expression leading to stress tolerance, but reduced growth is inevitable event even in the condition without stress, for example minimum 12 genes in Arabidopsis can be activated by DREB1A, consequently plant would be tolerant and dwarfism (Bahmani *et al*., 2015). Salinity-induced ROS formation can lead to oxidative damages in various cellular components such as proteins, lipids, and DNA, interrupting vital cellular functions of plants (Gupta and Huang, 2014).

Percentage of surviving callus and weight of callus

All calli used in this study was still survived until the end of stress periods. Complete data resilience callus on salinity stress can be seen in Table 1. The data shows that the percentage of surviving callus Talango varieties in 0, 2500, and 5000 ppm were 100%, but in 7500 ppm the surviving percentage of callus were reduce to 66.7%. This is in accordance with the observation of callus morphology (Figure 2), which Talango callus grown on 7500 ppm NaCl medium cannot survive under high salinity stress conditions and the color significantly become browning. While the percentage of surviving callus of Manding variety in all treatments are 100%. Different percentage of surviving callus in salinity stress can be influenced by various defense mechanisms of certain callus.

The measurement callus weight can be seen in Table 2 .The data in Table 2 shows that the higher levels of NaCl a callus can reduce the weight or just stagnant. Moreover, there are several a callus that shows dry symptoms and eventually die. The weight of a callus on the control can be reach 2 times compared with the early weight because the cell is continously in mitotic condition without any differentiation. In 7500 ppm, callus were lost its water since the concentration of ion in extracellular higher than in intracellular.

After selected into NaCl medium, the surviving callus were isolated its DNA in term to know the varian induced over this research. Bordallo *et al* in 2004 carried out 20 arbitrary sequence primers to evaluate the somaclonal variation among five of potato cultivars.It indicated a high level of genetic variation among cultivars.

Table 1. Percentage of surviving callus (%)

Varieties	Surviving callus			
	A	B	C	D
Talango	100	100	100	66,7
Manding	100	100	100	100

Notes: A= 0 ppm NaCl; B= 2500 ppm NaCl; C= 5000 ppm NaCl; D= 7500 ppm NaCl

Table 2. Effect of NaCl on weight of callus

Varieties	NaCl Concentration (ppm)	Average ± SE (mg)
Talango	0	13.33 ± 1.39
	2500	6.67 ± 1.39
	5000	3.33 ± 1.39
	7500	0.00 ± 0.00
Manding	0	996.70 ± 6.22
	2500	370.00 ± 3.81
	5000	173.30 ± 3.67
	7500	170.00 ± 0.00

Notes: SE (*Standart error*).

The differences on percentage of surviving callus in salinity stress can be influenced by various defense mechanisms callus. Some tolerance mechanisms to protect against the effects of salinity stress by the synthesis of various compounds such as proline, sucrose, trehalose, homeostasis ion, and induction of antioxidant enzymes (Rai, 2011). Furthermore the substances can limit the absorption of salt and adjust the osmotic pressure. Mansour *et al*. (2005) concluded that tolerance mechanisms in plants *Z. mays* against the salinity stress by increasing the accumulation of proline and glycine betaine on the tissue. Based on this result, it inferred that the percentage of surviving callus with a value of 100% is influenced by their tolerance mechanisms.

Evaluation of callus diversity using RAPD technique

Various technique and methods were also well developed to select the best candidate for breeding purpose. Several analysis including morpho-physiological, biochemical, cytological and DNA-based molecular markers approaches are the most common methods to select the germ plasm. Molecular marker had been developed to ensure the genetic variation among varieties. Somaclonal variation can pose a serious problem in any micropropagation program, where it is highly desirable to produce true-to-type plant material (Krishna *et al*, 2016). One of the easiest and low

cost of molecular marker is Random Amplified Polymorphic DNA (RAPD).

RAPD method were used to analyze genetic variation in callus exposed to NaCl. The visualization of DNA bands from RAPD technique can be seen in Figure.3 and 4. RAPD analysis using 10 random primers with a length 10 basepairs (bps). Primers used are OPA 02, 0PA10, 0PA 13, 0PB 13, OPB 07, OPC 02, OPD 08, OPI 01, OPK 20, OPU 19, and OPU 20. The results showed that out of 10 primers used only 5 primers showed polymorphism in two varieties, namely 0PA 13, OPB 07, OPC 02, OPK 20, and OPU 19. While 5 other primers namely, OPD 08 and OPI 01 only able to show polymorphism in Talango variety. On the other hand OPA 02, OPA 10 and OPU 20 were unable to produce polymorphism in Manding variety. Amplification products in two varieties ranged from 250 bp to 1500 bp.

Polymorphisn can be show by the difference amplification of DNA fragments that observed and scored as the presence or absence of differences in sequence, thereby indicating a genetic variation in the DNA chain (Ruwaida, 2009). Visualization of amplified DNA bands (Figure 3 and 4) shows the differences in the number and positions of DNA bands between callus in the control and the treatment. This condition suggests that there is a polymorphism in callus treated stress. The presence of the polymorphism indicates that there is a genetic difference between the control callus and tolerance. Polymorphism shows the variation of genotypes character callus derived from in vitro selection.

In Talango variety, OPA 13 primer produces two bands in control and one band only in 7500 ppm. Primer OPB 07 produces three bands in control and two bands in 7500 ppm. OPC 02 has three bands while in 7500 ppm produces one band only. OPD 08 produces four bands and three bands in 7500 ppm. OPI 01 produces one band and no band appears in 7500 ppm. OPK 20 has three bands each in control and treatments but the position of those bands are different it means that the primers amplify different number of bases in plant genome. OPU 19 produces four bands in control and three bands in 7500 ppm. Three other primers namely OPA 02, OPA 10 and OPU 20 gave the same patterns of bands products in control and 7500 ppm.

In Manding variety, primer OPA 02 produces two DNA bands in control and one band produce in 7500 ppm. Primer OPA 10 produces one bands in control, but in 7500 ppm, this pri-

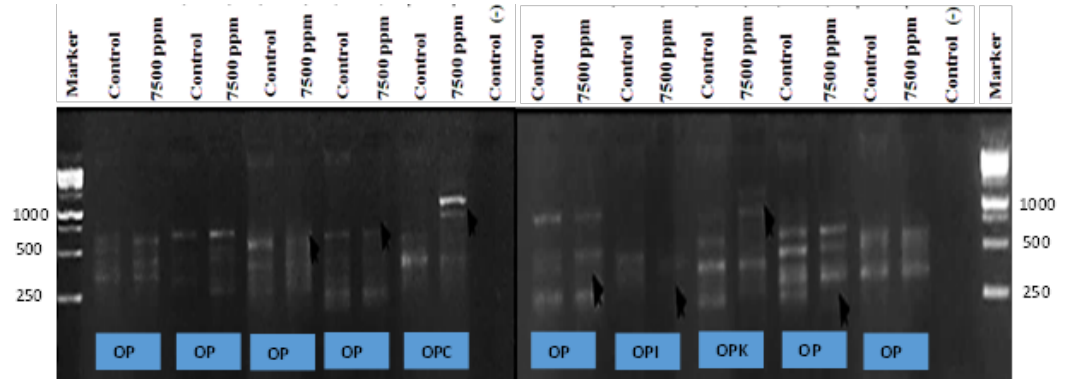


Figure 3. Visualization of DNA bands are amplified by varieties Talango (arrows indicate polymorphic DNA bands).

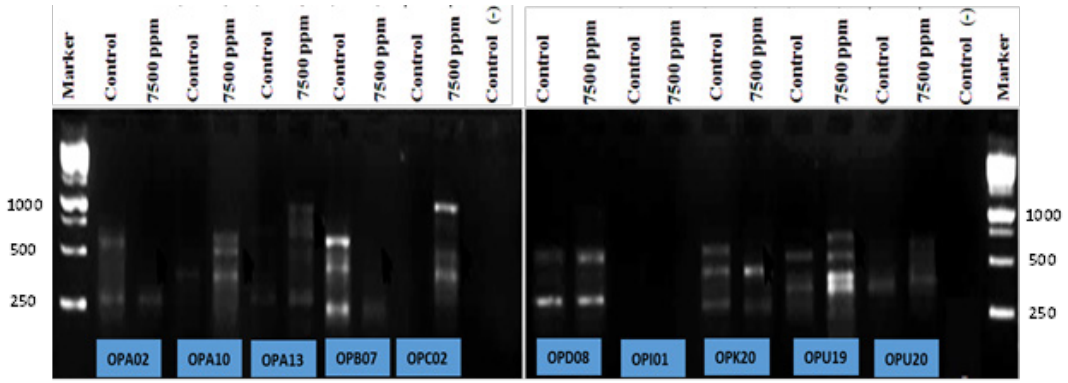


Figure 4. Visualization of DNA bands are amplified by varieties Manding (arrows indicate polymorphic DNA bands).

mer can produce three bands or two new bands were appears. Primer OPA 13 produces one bands in the control and shows polymorphism due to appearance of two new DNA bands in 7500 ppm treatment. Primer 07 OPB produces three bands and one band only in 7500 ppm. OPC 02 does not produces the DNA bands in control, but in 7500 ppm treatment appeared three new DNA bands indicating the occurrence of polymorphism. Primer OPK 20 produces 3 bands in control and 2 bands in 7500 ppm. Primer OPU 19 shows a polymorphism as four bands in control were appears and 3 bands in 7500 ppm. OPD 08 produces two DNA bands in both treatments, but did not show polymorphism. OPI 01 does not produces the DNA bands in both treatments. Primer OPU 20 were also has the same position and number of band in control and 7500 ppm.

The results showed that there is a primer that cannot produce DNA bands or no amplification product. It can be caused no complementary sequences in genomic DNA or there is only one strand of DNA containing sequences complementary to the primer (Figure 3). The quality of DNA were also a limiting factor over the presence of bands. In fact, the concentration of DNA template that is too small can cause DNA bands is not amplified (Sunandar & Imron, 2010).

CONCLUSION

The callus induction is an effective method to increase the diversity of maize. MS medium supplemented with 4 ppm of 2.4 D was the best medium to induce the callus formation. All calluses with various type of genetic character selected in selection medium while the higher levels of NaCl gave a negative effect on callus growth. The weight of a callus on the control might reach 2 times compared to its early weight, since the cells were continue to actively mitotic without any differentiation. Moreover, in 7500 ppm callus was dry because the cells in crenation condition. The surviving calluses were isolated its DNA genome to observe the differentiation between initial callus and surviving callus in 7500 ppm of NaCl. Out of ten RAPD primers, only five primers performed polymorphisms i.e OPA 13, OPB 07, OPC 02, OPK 20, and OPU 19 in those two varieties. Those five primers were important as molecular marker in salinity tolerant maize. New clone or variant from Manding variety shows better tolerance under salinity stress.

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