THE TOLERANCE OF IN VITRO SELECTED AND UNSELECTED SOMACLONES OF PEANUT TO OSMOTIC STRESS CAUSED BY POLYETHYLENE GLYCOL

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ABSTRACT
The research was aimed at comparing the tolerance level against osmotic stress in vegetative phase of in vitro selected and unselected somaclones of peanut and estimating the tolerance mechanism. The materials were peanut somaclones regenerated from somatic embryo as a result of in vitro selection in a medium containing polyethylene glycol 6000 (PEG-6000) 15%, peanut somaclones regenerated from unselected somatic embryo, and peanut plants grown from seeds as the standard plants. The osmotic stress treatment was given by watering PEG-6000 15% solution every two days until 7 weeks. As a control group were the plants watered with optimum water every two days. The result showed that the percentage of tolerant plants in the selected somaclones was higher than that the unselected somaclones. The tolerance mechanism of somaclones against osmotic stress caused by PEG-6000 was not related to the roots/shoot ratio and primary root length, and this phenomenon differed from standard plants.

Keywords: Peanut, Osmotic tolerance, Somaclonal variation, in Vitro selection, Polyethylene glycol

INTRODUCTION
Tissue culture is an alternative tool for developing stress-tolerant cultivar and could be conducted under controlled conditions with limited space, time, and cost. The culture could induce variation in regenerated plants and useful for crop improvement. The genetic variation among such plants is called somaclonal variation, and the obtained plant with somaclonal variation in certain trait is called somaclone (Karp, 1994). The somaclonal variation provides a valuable source of genetic variation for desired traits, including disease resistance, abiotic stress tolerance, and quality and quantity yield improvement (Predieri, 2001; Matheka et al., 2008; Miguel and Marum, 2011).

Somaclonal variations occur at many characteristics of plant randomly either be desired or not. In vitro selection using medium culture containing selective agents offers the chance to select plants with desirable characteristics (especially stress tolerance) because only explant tolerating high level of selective agents will survive. The selective agent must simulate the stress condition in the field (Mohammed et al., 2000; Rai et al., 2011). The explants are exposed to a broad range of the selective agent supplemented to the culture medium, and then the explants capable of sustaining such environments are selected.

Drought is one of abiotic stress involves the absence of rainfall for a period of time, long enough to cause water deficit with a decrease of water potential in plant tissues (Mundree, 2002; Molnar et al. 2004). The selecting agent usually used in in vitro selection for drought is polyethylene glycol (PEG). PEG of high molecular weight is a non-penetrating osmoticum which lowering water potential of nutrient solutions without being taken up or being phytotoxic. Many studies suggest that PEG of 6000 molecule weight (PEG-6000)
is the suitable selective agent to select drought-tolerant variation somaclones (Weele et al., 2000; Ragab et al., 2007; Bidabadi et al., 2011). The PEG sub-lethal concentration in the selective medium is thought efficiently select the living capability of somatic embryo in low osmotic potential. The somatic embryo capable of living in the selective medium are expected to have tolerant characters to cope the lack of water, and the regenerated plants from these somatic embryos are expected to be tolerant to drought (Mohammed et al., 2000).

Nevertheless, plants improvement through in vitro selection has some limitations, for example reducing of somatic embryo induction, plantlet regeneration, and correlation between the trait mechanisms appeared in cultured cell and those of the whole plants. There are parallel decreases of embryogenic callus induction and plant regeneration efficiency from selected callus with increasing concentration of PEG used in selection medium of rice (Al Bahrany, 2002), maize (Matheka et al., 2008), tomato (Aazami et al., 2010), and durum wheat (Soliman and Hendawy, 2013). It is believed to be caused by increasing osmotic stress due to the addition of PEG in the medium is accompanied by a sharp decrease in water content of tissues (Heyser and Nabors, 1981) or alter DNA sequence (Arumingtyas et al., 2012).

Therefore it is necessary to determine the effectiveness of in vitro selection using PEG in drought resistance improvement of crops. This research was aimed at comparing the growth response and the tolerance level against PEG stress on vegetative phase of in vitro selected and unselected somaclone peanut plants, and estimating the tolerance mechanisms.

METHODS

Plant materials

Three populations of peanut cv. Kelinci, i.e. in vitro selected somaclone, in vitro unselected somaclone, dan standard plant were used for this study. The in vitro selected somaclone were obtained through culturing somaclonal variant of somatic embryos (SEs) in selective medium (Fig. 1A), that was liquid Murashige and Skoog (MS) medium containing sub-lethal concentration (15%) of PEG 6000 (Rahayu et al. 2006) for 3 months and were sub-cultured each month. The insensitive SEs in the selective medium (Fig. 1B) were isolated and shifted into solid MS-P16 (MS medium supplemented with picloram 16 μM and active charcoal) for 2 months for proliferation. Further the SEs were regenerated at the standard way until R2 generation, and called R2-K15 plant population. The in vitro unselected somaclones were derived from culturing somaclonal variant of SEs in MS-P16 medium for 3 months and were sub-cultured each month, and then regenerated through the same way with the in vitro selected somaclones until R2 generation, and called R2-K0 population. To get the standard plants, two seeds were planted on each pot. One week after planting the selection was made by keeping one plant showed similar in growth in each pot while the other plant was removed.

![Figure 1. In vitro selection of somatic embryo variant (SEV) in liquid MS medium added by PEG 15%. A. Performance of SEV after 1 month cultured in selective medium; B. Performance of SEV after 3 months cultured in selective medium showed whitish insensitive SEV; C. Roots growth of standard (left), R2-K0 (center) and R2-K15 (right) plants after PEG application](image)

Experiment design

This research was conducted in the glass-house of Genetic Resource Research Center, Bogor, Indonesia. The experiment was carried out in a completely randomized design with two factor, that were stress treatment at two levels (PEG stress and non-stress), and plant population at three levels (R2-K15, R2-K0 and standard plants). The experimental unit was one pot with a peanut plant at age of one month. Each treatment was replicated 20 times.
Preparation of planting medium in the glasshouse

Mixture of charred husk and manure at a ratio of 1:1 (v/v) as planting medium was filled in the 600 ml plastic pots with the height of 30 cm and diameter of 10 cm, and then covered with black polyethylene plastics. Each pot was filled with planting medium as much as 500 g or equivalent to approx. 25 cm high, then watered and left to dry to help ease the water absorption during PEG treatment. The planting medium were then watered with 6-10 g/l NPK fertilizer solution as much as 300 ml per pot or until field capacity is reached, and then 10-15 pills of slow-released NPK fertilizer were added in each pot.

PEG Application

PEG solution was prepared by adding 150 g PEG 6000 crystals in water until reaching the volume of 1 l solution. PEG solution was then poured into the pot as much as 30 ml every two days since the plant had four perfectly opened leaves and the procedure was repeated until week 7. For non-stress control group, optimum condition was given by watering plain water at the same amount to the pots. After four weeks of watering, the volume was increased to 40 ml per pot. The pots were placed in several rows with an interval of 0.1 m between pots in a row and an interval of 0.2 m between rows.

Data analysis

The responses to be examined were intensity of leaf damage (on week 4 post-treatment) and plant growth. The plant growth variables measured were number of leaves, dry shoot weight, length of primary roots, and dry root weight at seven weeks post-planting. At the measuring time, the plants were taken carefully from the pots by ripping the plastic pots to reveal the planting medium in order to get the whole roots. The plants were then washed under the running water to clean the planting medium stuck on the roots (Fig. 1C). The roots and shoot were kept in an oven at 80°C for 3 days to obtain dry roots and shoot.

Data collected from each treatment were statistically analyzed for mean and standard deviation using MS-EXCEL software. Stress and population effect on quantitative character were evaluated by analyzing the data by two-ways analysis of variance, and significant differences among population means were compared by Duncan's Multiple Range Test at confidence rate of 5% using SAS (statistical analysis system) version 9.0.

![Figure 2. Scores of leaf damage of peanut plant cv. Kelinci due to PEG 15% stress on charred husk medium in the glasshouse. Score 0: no visible chlorosis nor necrosis symptom; score 1: chlorosis on the edge of the leaves up to around 10% of the leaf area; score 2: chlorosis on the edge of the leaves 10-30% of the leaf area; score 3: chlorosis on edge until middle part of the leaves 30-60% of the leaf area; score 4: chlorosis for more than 60% of the leaf area and or some necrosis.](image)
Table 1. Criteria to determine the response of the plants based on leaf damage intensity (LDI) value

<table>
<thead>
<tr>
<th>Plant response</th>
<th>LDI</th>
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<tbody>
<tr>
<td>Very Tolerant</td>
<td>No damage, intensity of damage is 0%</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Slight damage, intensity of damage is 0% ≤ 5%</td>
</tr>
<tr>
<td>Relatively Tolerant</td>
<td>Mild damage, intensity of damage is 5% &lt; x ≤ 10%</td>
</tr>
<tr>
<td>Relatively Sensitive</td>
<td>Mild damage, intensity of damage is 10% &lt; x ≤ 25%</td>
</tr>
<tr>
<td>Sensitive</td>
<td>Heavy damage, intensity of damage is 25% &lt; x ≤ 50%</td>
</tr>
<tr>
<td>Very Sensitive</td>
<td>Heavy damage, intensity of damage is &gt; 50%</td>
</tr>
</tbody>
</table>

To estimate the drought tolerance mechanism of somaclones, coefficient of correlation between LDI and root growth (namely primary root length and root/shoot ratio) was calculated. Root growth variable was choice because generally plants capable of tolerating drought stress would have deeper rooting system (Setiawan, 1998), higher roots growth rate (Molnar et al., 2004) or larger lateral root number (Badianne et al., 2004).

RESULTS AND DISCUSSION

Overall, in both optimum and stress conditions, ranges of value of leaves number, dry weight of shoot, dry weight of roots, and length of root of R2-K0 and R2-K15 populations were wider than that standard population (Figs. 3, 4, 5, 6). The wider range of the value of a character in a population than that in standard population showed the somaclonal variation in certain character of such population. The character value which was lower than that the standard was called negative variation, and the reverse was called positive variation.

Under optimum condition, one line of R2-K0 and one line of R2-K15 were negative variation somaclones for leaves number variable (Fig. 3), two lines of R2-K0 and two lines of R2-K15 were negative variation somaclones, and two lines of R2-K0 and one line of R2-K15 were positive variation somaclones for dry shoot weight (Fig. 4). Under stress condition, there is no variation somaclones of leaves number (Fig. 3), and one line of R2-K0 were positive variation somaclones for dry shoot weight (Fig. 4).

Under optimum condition, one line of R2-K0 and one line of R2-K15 were negative variation somaclones for leaves number variable (Fig. 3), two lines of R2-K0 and two lines of R2-K15 were negative variation somaclones, and two lines of R2-K0 and one line of R2-K15 were positive variation somaclones for dry shoot weight (Fig. 4). Under stress condition, there is no variation somaclones of leaves number (Fig. 3), and one line of R2-K0 were positive variation somaclones for dry shoot weight (Fig. 4).

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Under optimum condition, two lines of R2-K0 and one line of R2-K15 were positive variation somaclones for dry root weight (Fig. 5), one line of R2-K0 and one line of R2-K15 were negative variation somaclones, and one line of R2-K0 and two lines of R2-K15 were positive variation somaclones for root length (Fig. 6). Under stress condition, two lines of R2-K0 and one line of R2-K15 were positive variation somaclones for dry root weight (Fig. 5), and one line of R2-K0 and one line of R2-K15 were positive variation somaclones for root length (Fig. 6). These results showed that the probability of somaclonal variation evidence in the R2-K0 population was not different from the R2-K15 population. It could be expected to obtain dehydration tolerance variant from the standard plant.

**Figure 6.** The frequency distribution of root length on standard (■), R2-K0 (□), and R2-K15 (△) populations under optimum and PEG 15% stress conditions. Range of root length A (x<9.2), B (9.2≤x<15.9), C (15.9≤x<22.6), D (22.6≤x<29.3), E (29.3≤x<36)

**Tolerance against PEG stress**

Based on LDI, all of plants of the standard population were categorized as relatively tolerant to PEG stress, whereas the plant of R2-K0 population segregated into eight sensitive lines, 10 relatively tolerant lines, and two tolerant lines; and the plant of R2-K15 segregated into four sensitive lines, 10 relatively tolerant lines, and two tolerant lines (Table 2).

The *in vitro* selected somaclone and unselected somaclone both produced plants with more tolerance to stress due to PEG compared to the standard plants, but the percentage of tolerant plants in the *in vitro* selected somaclone (30%) was higher than that the *in vitro* unselected somaclone (10%). This finding is supported by previous data, i.e. the decreasing of shoot growth and root fresh weight due to PEG stress of R2-K15 (*in vitro* selected somaclone) were lowest compared to R2-K0 (*in vitro* unselected somaclone) and standard plants (data not shown).

<table>
<thead>
<tr>
<th>Response against PEG 15% stress</th>
<th>Number of plants of population</th>
</tr>
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<tbody>
<tr>
<td>Sensitive</td>
<td>0</td>
</tr>
<tr>
<td>Relatively tolerant</td>
<td>20</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0</td>
</tr>
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</table>

**Mechanism of tolerance against PEG stress**

There were significant correlation between LDI and root/shoot ratio and primary root length of plants of standard population, otherwise no correlation between LDI and root/shoot ratio and primary root length of plants of R2-K0 and R2-K15 populations. The determination coefficient ($R^2$) values between LDI and root/shoot ratio and primary root length of standard population were 0.612 and 0.465, respectively; whereas in the R2-K0 population the $R^2$ were very small, i.e. 0.007 and 0.026, respectively; and in the R2-K15 population were 0.118 and 0.068, respectively (Fig. 7). Based on these results, it could be said that the tolerance against PEG stress on the standard plant population was related to root growth, while on the somaclones populations were not related to the root growth.

These data revealed that among the factors of drought resistance of peanut standard plants, primary root length is a dominant factor which determined 61.2% of drought resistance variation. Separately roots:shoot ratio was also a fairly dominant factor of the drought resistance change, around to 46.5%. In R2K0 population, primary root length and roots:shoot ratio just determined 0.7% and 2.6% respectively, and in R2K15 population the both factors determined only 1.18% and 6.8% of the variation in resistance to drought.

Based on these results, it could be said that the tolerance against PEG stress on the standard plant population was related to root growth. This result was in line with the previous studies which showed that plants capable of tolerating drought stress would have deeper rooting system (Setiawan 1998), higher roots growth rate (Molnar et al. 2004) or larger lateral root number (Badianne et al., 2004). The tolerance against drought stress through the intensive root growth basically is the escape mechanism which often impacts negatively on crops (Mundree et al., 2002). The intensive root growth requires more biomass, which might be more advantageous if allocated to pod development. Therefore,
it would be advantageous if plants develop tolerance not through intensive root growth, because it will not reduce the crop. It is suggested that further research is conducted to understand the mechanism of tolerance against drought stress on the lines resulted from in vitro selected and in vitro unselected populations.

In contrast, the drought tolerances on the somaclone populations (R2K15 and R2k0) were not related to the root growth. In other words it can be stated that differ from the standard plant, the root growth of somaclones were not became the determining factor of plant tolerance against drought stress. The ratio of roots/shoot and root length did not show significant correlation with plant’s tolerance against PEG stress. This suggested that tolerance against drought stress on plants of in vitro selected and in vitro unselected populations did not occur through root growth intensive root. This mechanism should be advantageous because it would not reduce the crop (Mundree et al. 2002), and it would be worth to evaluate this in the future.

Preparing in vitro cultures with water stress agents was efficient in selecting a drought tolerant plant. In vitro screening with induction of chemical drought using PEG 6000 to examine water stress tolerance could be a proper track to develop drought-tolerant lines (Ragab et al., 2007; Bidabadi et al., 2011).

All of the results of this study indicated that the induction of somaclonal variation and followed by in vitro selection using 15% PEG 6000 effectively induced drought resistance enhancing and the mechanisms was differ from the standard plant. The problem of loss of regeneration ability during in vitro selection can be overcome by the use of explants with high morphogenic potential which may ensure successful regeneration (Rai et al., 2011). Epigenetic adaptation is another obstacle for the selection of rare mutants with true tolerance, i.e. meiotically inherited, which can be prevented by the use of short-term or one-step selection (Miguel and Marum, 2011).

**CONCLUSION**

Probabilities of somaclonal variation evidence on some morphological traits i.e. number of leaves, dry shoot weight, length of primary roots, and dry root weight of in vitro selected somaclones were not different from the unselected somaclones. There were positive and negative variants in selected and unselected somaclones. From the selected somaclone and unselected somaclone populations there were plants with more tolerant to stress due to PEG compared to the standard plants, and the percentage of tolerant plants of selected somaclone (30%) was higher than that the unselected somaclone (10%). Differed from the standard plant, the mechanisms of peanut somaclones tolerance against PEG stress were not related to the ratio of roots/shoot and primary root length.

**REFERENCES**


