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by Jurnal_pa Widi Buat Gb_7

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RESEARCH ARTICLE

REVISED Selection and regeneration of purple sweet potato calli against drought stress simulated by polyethylene glycol [version 2; peer review: 1 approved]

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Abstract

Background: Water shortage due to natural and/or technical drought stress, widespread throughout Sumatra, Java, Sulawesi and Kalimantan islands, significantly reduces crop production. The development of varieties tolerant to drought stress is important since it is more effective rather than improving irrigation infrastructure to increase the sweet potato productivity.
Methods: Selection and regeneration experiments assessing purple sweet potato callus tolerance of drought stress, simulated by polyethylene glycol (PEG), were conducted to generate new variant plants tolerant of drought stress. Sterile explants (leaf and petiole) generated from previous *in vitro* culture were inoculated to the Murishage and Skoog (MS) medium containing plant growth regulator combination as treatments to induce calli. The calli were then transferred to half-MS medium containing 0, 5, 10, 15 and 20% PEG as selection agent for drought tolerance. The surviving calli were regenerated in the MS medium containing 0, 0.5, 1 or 1.5 mg l^{-1} 6-benzylaminopurine (BAP). The callus formation, growth and survivability during *in vitro* culture were measured.
Results: Calli were successfully formed in almost all media containing *2,4-Dichlorophenoxyacetic acid* (2,4-D) with the concentration of 1, 2, 3 and 4 mg l^{-1} and BAP (concentration: 0.5 and 1 mg l^{-1}), but the medium of MS + 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP resulted in the highest number of induced calli per treatment (mean=11.36), with the percentage of responsive explants standing at around 96%. The higher the concentration of PEG, the lower the number of surviving calli. At 20% PEG, only 54.42% calli survived. There were five plants successfully regenerated from the survived calli at 20% PEG, using MS medium containing 1.5 mg l^{-1} BAP.
Conclusions: The experiment has successfully produced putative drought-tolerant plants by callus screening using PEG as drought-tolerance-selecting agent in purple sweet potato.

Keywords

Callus formation, purple sweet potato, drought tolerance, Polyethylene Glycol, in vitro selection

Open Peer Review

Reviewer Status ✔

	Invited Reviewers
	1
REVISED **version 2** published 28 Mar 2019	✔ report
version 1 published 03 Jan 2019	? report

1 **Yosep Seran Mau**, University of Nusa Cendana, Kupang, Indonesia

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the ICTROPS 2018 collection.

Corresponding author: Widi Sunaryo (widi_sunaryo@yahoo.com)

Author roles: Sunaryo W: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Writing – Original Draft Preparation, Writing – Review & Editing; **Darnaningsih D**: Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization; **Nurhasanah N**: Conceptualization, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision

Competing interests: No competing interests were disclosed.

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REVISED **Amendments from Version 1**

1. We have added 9 more recent literature references (within last 10 years) in this new version.
2. We have made revisions for the wrongly written (40% PEG) in certain texts, and replaced it with the true PEG concentration (20%) throughout the text.

See referee reports

Introduction

Drought stress is a major limiting factor in increasing the production of several important crops in a large number of regions in Indonesia. The effect of drought stress on plant growth is largely determined by the amount of stress the plant is exposed to and the growth phase the plant was in during drought stress. In addition, drought stress causes inhibition of plant growth and plant roots[1,2] and as a consequence, plants will grow slowly[3] and their productivity is severely reduced[4]. Development of cultivated plants tolerant to drought stress, including sweet potato, is an important approach to solving water shortage issues[5].

In vitro selection is a breeding strategy widely used to produce new variant plants that are resistant or tolerant to disease, herbicides or extreme environmental stresses, including drought stress[6]. This method selects genetic variation arising from tissue culture processes especially produced from natural or artificial mutation[7]. The rapid multiplication of undifferentiated cells during callus formation increases the possibility of natural mutation due to rapid cell division[8]. Such genetic changes are subsequently selected as useful traits in breeding programs.

Polyethylene glycol (PEG) solution can be used as drought-tolerant selecting agent in soybean[9] and other plants[10–12]. PEG is able to control the decrease of water potential homogeneously, therefore it can mimic the potential of groundwater[13]. The long-term use of PEG will not cause cell damage, because PEG has molecular weight of more than 6000 g/mol that cannot be absorbed into plant tissues[14]. This research attempted to produce new variant of purple sweet potato tolerant to drought stress via *in vitro* selection using PEG as a selection agent.

Methods

Callus growth

Young leaves and petioles from previous *in vitro*-generated plants grown using standard Murishage and Skoog (MS) medium[15] were used as explants. The intact leaf were sliced into 1 cm^2 of lamina and 1 cm length of petiole. The explants were then inoculated to the MS containing plant growth regulator of 2,4-dichlorophenoxyacetic acid (2,4-D) (Merck, Cat. No.31518, Germany) combined with 6-benzylaminopurine (BAP, Merck, Cat. No. B3408, Germany) to induce calli. The explants were grown and placed at a sterilized 600 ml bottle containing around 20 ml solidified medium for a month at the culture room at a temperature of 25±2°C. The composition of the treatments were Z_1 (MS + 0 mg L^{-1} 2,4-D + 0 mg L^{-1} BAP), Z_2 (MS + 1 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP), Z_3 (MS + 2 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP), Z_4 (MS + 2 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP), Z_5 (MS + 3 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP), Z_6 (MS + 3 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP), Z_7 (MS + 4 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP), and Z_8 (MS + 4 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP). All treatments were replicated five times. In total there were two types of explants x eight treatments x five replication x five explants per replication/bottle = 400 experiment units.

Assessment of callus growth

The green, fresh and compact calli produced at the first experiment were selected and transferred to MS medium with half the usual level of nutrients containing 0, 5, 10, 15 and 20% PEG as selection agent medium of drought tolerance. All treatments were replicated three times. In total there were five treatments x three replications x four explants per replication/bottle = 60 experiment units. The surviving calli in the PEG selection-agent medium were regenerated in MS medium containing 0, 0.5, 1 or 1.5 mg L^{-1} BAP to induce shoots and roots. The growth variables during *in vitro* culture, such as the number of calli induced per treatment, percentage of responsive explants to induce calli, percentage of surviving calli under drought stress simulated by polyethylene glycol, and number of regenerated plants, were observed.

Results and discussion

Callus production

All media applied in the experiment could successfully induce callus formation except the basal medium containing no growth regulator (control). The existence of 2,4-D and BAP in various concentration combination was able to induce callus formation but the number of callus per explant and the percentage of responsive explant were varied (Table 1, Figure 1A, B). The highest number of induced calli per treatment was observed using the Z_3 treatment medium. These results were observed using both leaf and petiole (Figure 1A, B). Callus formation was successfully induced in sweet potato using both explants[16,17]. The use of 2,4-D to induce callus formation is common[16,18]. Callus formation can be induced by other plant growth regulators such as zeatin, or 1-naphthaleneacetic acid combined with gibberellin (GA_3)[17,19,20]. These calli emerged and was developed from the mesophyll cells in the leaf and protoplast from the stem or petiole[16,17].

Drought-stressed callus growth

The fresh, green and compact calli produced in the first experiment was transferred to the drought stressed medium simulated by MS medium containing different concentration of PEG to discover the putative drought tolerant calli of purple sweet potato. At least one callus survived in all PEG-containing medium, but the survival rate was varied (Table 2, Figure 1C). The higher the concentration of the PEG in the medium the lower survival rate of the calli in the selection medium. This indicates that drought stress caused by PEG simulation affects callus survivability and can be used as drought-tolerant selection medium, as also reported in other crop plants such as soybean[21–25], eggplant[26], sunflower[13], grass[27,28], rice[29,30], tobacco[31] and shorgum[32]. The putative drought-tolerant plants are screened

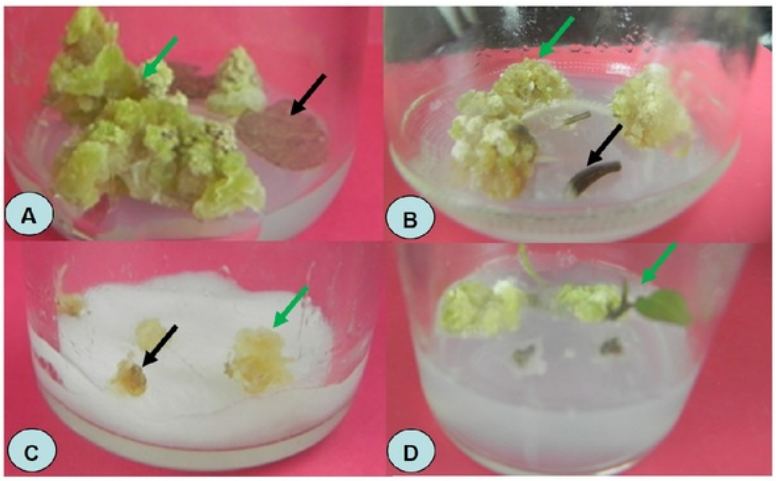

Figure 1. Callus selection and regeneration for drought tolerant in purple sweet potato. (**A**) Callus induction from leaf explants (black arrow shows irresponsive explant; green arrow shows the responsive explant). (**B**) Callus induction from petiole explants (black arrow shows unresponsive explant; green arrow shows the responsive explant). (**C**) Callus selection for drought tolerant using polyethylene glycol (black arrow shows dead explant; green arrow shows the survived explant). (**D**) Callus regeneration (green arrow shows the initiated shoot from the callus).

Table 1. Callus induction using two different explants of purple sweet potato. Values shown are mean ± standard deviation unless indicated.

Callus induction medium	Leaf		Petiole	
	Calli induced per treatment	Responsive (%)	Calli induced per treatment	Responsive (%)
Z_0 (MS + 0 mg L^{-1} 2,4-D^1 + 0 mg L^{-1} BAP)	0.00 (± 0.00)	0	0.00 (± 0.00)*	0
Z_1 (MS + 1 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP)	8.80 (± 1.94)	68	8.76 (± 2.09)	76
Z_2 (MS + 1 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP)	10.44 (± 2.01)	92	10.44 (± 1.41)	88
Z_3 (MS + 2 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP)	11.36 (± 2.25)	96	11.32 (± 2.62)	64
Z_4 (MS + 2 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP)	5.12 (± 2.00)	92	5.12 (± 3.04)	76
Z_5 (MS + 3 mg L^{-1} 2,4-D^1 + 0.5 mg L^{-1} BAP)	5.28 (± 0.83)	88	5.28 (± 1.04)	44
Z_6 (MS + 3 mg L^{-1} 2,4-D^1 + 1 mg L^{-1} BAP)	7.40 (± 1.98)	92	7.40 (± 1.36)	88
Z_7 (MS + 0 mg L^{-1} 2,4-D^1 + 0 mg L^{-1} BAP)	2.32 (± 1.75)	72	2.32 (± 2.20)	88
Z_8 (MS + 0 mg L^{-1} 2,4-D^1 + 0 mg L^{-1} BAP)	8.60 (± 0.80)	84	8.60 (± 1.86)	92

MS, Murishage and Skoog medium; BAP, 6-benzylaminopurine.

from regenerated plants derived from the survived calli in the highest concentration of PEG[33]. There was no significant different response between calli derived from the leaf and petiole (Table 2).

The surviving calli grown in medium containing 20% PEG were incubated in the regeneration medium to initiate shoot and/or root. The only medium MS containing 1.5 mg L^{-1} BA can successfully induce shoot and root growth (Table 3, Figure 1D). This indicates that the calli exposed with high concentration of

Table 2. Percentage of surviving purple sweet potato calli under drought stress simulated by different concentration of polyethylene glycol (PEG).

Explant	PEG Concentration (%)				
	0	5	10	15	20
Leaf	90.11	89.85	88.37	76.32	55.42
Petiole	96.88	86.88	81.67	76.98	57.92

Table 3. Initiation of shoot and root derived from calli that survived 20% polyethylene glycol growth.

Callus induction medium	Number of regenerated plants	
	Leaf	Petiole
K_0 (MS + 0 mg L^{-1} BAP)	0	0
K_1 (MS + 0.5 mg L^{-1} BAP)	0	0
K_2 (MS + 1.0 mg L^{-1} BAP)	0	0
K_3 (MS + 1.5 mg L^{-1} BAP)	1	4

MS, Murishage and Skoog medium; BAP, 6-benzylaminopurine.

PEG are still viable to regenerate into whole plant. The results increase the possibility to get a new purple sweet potato variant tolerant to drought stress as also reported in soybean[23,24].

The raw data for calli and explant growth are available on OSF[34].

Conclusions and outlook

Initial production of putative drought tolerant plants by callus screening using PEG as drought tolerant-selecting agent in purple sweet potato was successfully done in this experiment. Callus was successfully induced in MS medium containing many different combinations of 2,4-D and BAP. Drought-tolerant screening using PEG is generally effective since there was a strong indication that the increase of PEG concentration caused the reduction of the callus survivability. However, the high percentage of surviving calluses at the highest concentration of PEG (20%) may indicate that the drought stress applied in the experiment was not apparently sufficient to induce cell mutation. Therefore, a future experiment using higher concentrations of PEG could provide more tolerant calli and increase genetic mutations, especially with regards to drought tolerance. Genetic evaluation and field experiments using water shortage treatment experiments will be the next investigation to clarify the genetic mutations involved and the stability of the drought-tolerance characteristics.

Data availability

The raw data on the number of calli formed per bottle for different treatment conditions and subsequent explant growth are available on OSF. DOI: https://dx.doi.org/10.17605/OSF.IO/DEGVY[34].

Grant information

The author(s) declared that no grants were involved in funding this work.

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Publisher Full Text

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Publisher Full Text

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http://www.doi.org/10.17605/OSF.IO/DEGVY

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Current Peer Review Status: ✔

Version 2

Reviewer Report 01 April 2019

https://doi.org/10.5256/f1000research.20423.r46413

✔ **Yosep Seran Mau**
Faculty of Agriculture, University of Nusa Cendana, Kupang, Indonesia

The authors had made revisions as suggested. Thus, I have no further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant breeding and plant protection

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 21 January 2019

https://doi.org/10.5256/f1000research.18579.r42525

? **Yosep Seran Mau**
Faculty of Agriculture, University of Nusa Cendana, Kupang, Indonesia

In general, the article is sufficient in both presentation and content, and provides new findings in the field. However, several of the following concerns, as parts of my review to the article, may need to be clarified by the authors:

1. *Does it cite the current literature?*

   Partly, as only about 25% of the references are within the last 10 years.

2. *Is the statistical analysis and its interpretation appropriate?*

   Partly, as there is inconsistency between the method and the results (analysis). In the method, the highest PEG concentration is 20% but in the results (Table 3) the highest PEG concentration is 40%, which is not consistent.

3. *Are all the source data underlying the results available to ensure full reproducibility?*

   Partly, in Table 3, there are four calli survived in the regeneration stage using K3 medium. If the 4 calli from petiole were all survived, then there must be 16 calli that have been regenerated in this stage, assuming that the same number of calli (four calli) was assigned into each growth medium. This does not match with the method and the results in Table 2, where only 57% calli (about 7 calli) survived the 20% PEG. 16 calli are needed for the four growth medium treatments in the regeneration stage. This needs to be clarified.

4. *Are the conclusions drawn adequately supported by the results?*

   Partly, for the following reasons:

   1) Inconsistency in the highest PEG concentration employed: 20% in the method and 40% in the results and conclusions.

   2) More than 50% calli survived at the highest PEG concentration may indicate mild to moderate stress intensity, thus, its effectiveness as in vitro selection of drought tolerance needs to be improved.

   3) There were only five calli survived in the regeneration stage (Table 3), which is very low in number as a starting genetic material for in vivo selection of drought-tolerant purple sweet potato clones.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant breeding and plant protection

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 16 Mar 2019

Widi Sunaryo, Faculty of Agriculture, Mulawarman University, Samarinda, Indonesia

Honourable reviewers
Thank you very much for the valuable comments in improving the scientific and writing quality for our manuscripts. We have carefully read and paid much attention for all comments, and directly made the required revisions according to the suggestions. Our responses concerning the comments from the reviewer are:

1. Thank you very much for the "Approved with reservations" status, we will make revisions as suggested.
2. We added 9 more recent pieces of literature (within the last 10 years) in the new version of the manuscript.
3. We have made revisions for the wrongly written (40% PEG) and replaced with the true PEG concentration (20%) in all the text (in the new version of the manuscript).
4. The survived calli in PEG medium (20%) at petiole explants were 57%. Each survived calli subsequently can be separated/divided into some smaller calli. This will provide enough calli for the next stage of the experiment (regeneration initiation). In the PEG selection medium, even in the highest concentration of PEG, the survived calli were still growing and larger. That is why the number of calli was still sufficient for the next stage of experiment.

Table 3 shows the number of plantlets (regenerated plants) and in the regeneration experiment. There was no selection (PEG selection) anymore.

1. Concerning this comment: "*More than 50% calli survived at the highest PEG concentration may indicate mild to moderate stress intensity, thus, its effectiveness as in vitro selection of drought tolerance needs to be improved*". We do agree with this suggestion, and we have put this suggestion in the conclusion and outlook part.
2. Since we have only 5 regenerated plants in the regeneration experiment, we will propagate (vegetatively) the plantlets to get a sufficient number of plant materials for the in vivo experiment. The in vitro propagation procedure for purple sweet potato is established and can be carried out very easily and fast.

Thank you very much for the comments. If there are still any mistakes or corrections, we are open for the next revision and improvement.

Competing Interests: None in writing

Comments on this article

Version 1

Reader Comment 11 Jan 2019

Duangporn Premjet, Center of Excellence in Research for agricultural Biotechnology, Faculty of Agriculture, natural Resources and Environment, Naresuan University, Muang, Phitsanulok. 65000, Thailand

Introduction:

Should add more literature on suitable PEG concentration to test for drought tolerance in related species.

Materials and Methods:

Adequate.

Results and Discussion:

Adequate.

General comments:

The research idea to develop drought tolerant purple sweet potato through in vitro selection by using stress is good and should have supporting funds to do more research until they get the whole plants.

Assoc.Prof. Dr. Duangporn Premjet,
Center of Excellence in Research for Agricultural Biotechnology,
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Competing Interests: No competing interests were disclosed.

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ORIGINALITY REPORT

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