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Efficient Method for Mass Propagation of Orchid (Vappodes Phalaenopsis) using Polyethylene Glycol (PEG) to Study the Induced Water Stress on the Relative Growth of Orchid Plants

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ABSTRACT

Tissue culture is a potent biotechnological technology that allows for the quick development of genetically homogenous plants with desirable features such as high yield, disease resistance, and long shelf life while using little water, space, and time. Drought stress is a significant abiotic element limiting worldwide agricultural yield. Orchid species, notably Vappodes phalaenopsis, are thought to be extremely susceptible to drought conditions. The current work investigated shoot, leaf, and callus induction in V. phalaenopsis under in vitro conditions, with a particular emphasis on the impacts of drought stress. To imitate drought stress, Murashige and Skoog (MS) medium was supplemented with 0.5 g/L polyethylene glycol (PEG-6000). Explants (shoot tips) grown in MS media with 2 mg/L BAP and 1 mg/L NAA showed optimum shoot initiation. Similarly, MS medium supplemented with 2 mg/L BAP, 1 mg/L NAA, and 0.5 g/L PEG induced maximum callus formation. The addition of PEG significantly reduced transpiration in orchid plantlets by decreasing the number of leaves and shoots, while simultaneously increasing callus induction. In contrast, MS medium without PEG supported enhanced shoot and leaf proliferation with reduced callus formation. These findings suggest that PEG-mediated osmotic stress alters morphogenic responses in

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orchids and may provide valuable insights into drought adaptation mechanisms under in vitro conditions.

Keywords: Polyethylene glycol (PEG), tissue culture, drought stress, BAP, NAA, orchids.

INTRODUCTION

In flowering plants, Orchidaceae is a large family, with 28,000 species and 763 genera distributed all over the globe, particularly in wet tropics. Orchids are known due to flowers and their long life span. The estimated world's flower trade is almost $4 billion (US (Zhang et al., 2018) The name Vappoddes *Phalaenopsis* comes from the Greek term phalaina, which means moth, and opsis, which means look-alike, and represents how the blooms appear to a flying moth. These orchids have a significant commercial value as cut flowers in the worldwide flower market. Phalaenopsis are the most popular orchids nowadays. According to statistical data from the Netherlands, Phalaenopsis market potential climbed from 5% to 66 percent. (Khatun et al., 2020.) It is the most profitable ornament in the world's floral market and has excellent value in the world's economic market. Therfore it is generally cultivated on larger scales (Fesya Salma Putri et al., 2019) Orchids are traded all over the world as cut flowers and potted plants. *Phalaenopsis* has a beautiful color that doesn't fade quickly and is the most popular cultivated Orchid globally. (Li et al., 2021). They are used mainly by society because of their low price and faster growth. They are also used in the medical industry because of their high polysaccharides, alkaloids, and other chemical components. With the expansion of economic globalization, the market demand for orchids has grown, including number and diversity, prompting scientists and breeders to create new kinds with unique looks, better resistance, and quality attributes. (Li et al., 2021b). In orchid cultivation, the main milestone is drought stress because of the low availability of water. (Fesya Salma Putri et al., 2019. Drought is a major abiotic limitation to plant production around the world. During drought stress, water absorption is complicated from roots. The efficacy of photosynthesis is also reduced to a greater extent due to stomata closure. To reduce transpirational water loss, which lessens the ability of leaves to fix carbon leading to early shedding of leaves. (Suis et al., 2015). Roots are the first organs that face drought stress and produce fine roots for deeper penetration in soil pores for better water absorption. (Basal et al., 2020). Drought/dehydration is one of the abiotic stressors that need to be avoided. Because the stress conditions may be easily manipulated, tissue culture can be used to evaluate

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tolerance under the conditions of an *in vitro* system. Furthermore, the minor variability in plant populations is observed over a short period by adopting an appropriate tissue culture technique (Ahmad et al., 2020a)

Due to human exploitation, orchids plants face conservation threats and are near extinction, so proper cultivation methods are required. Physiologically and morphologically, a lack of water can interfere with the growth of plants. (Untari & Puspitaningtyas, 2006). Two effective methods for drought stress are mannitol and Polyethylene glycol(PEG). Mannitol is non-toxic to plants, and PEG is inert to plants due to its molecular weight (Basal et al., 2020). PEG is the chemical that can lower the water potential and develop implanted network in plants against drought stress. (Untari & Puspitaningtyas, 2006). PEG is effective against drought stress simulation in plants due to its inert capabilities and less absorbance. (Basal et al., 2020). It is a non-ionic, inert polymer frequently used for imitating water stress conditions and is a well-established tool for genotype evaluation under laboratory circumstances. PEG induces osmotic stress, which lowers the photosynthesis rate. (Ahmad et al., 2020b). In the current study, physiological changes in response to PEG 6000 were investigated. According to our knowledge, there is no single study in which the root, shoots, leaves, and callus of *V.phelanopsis* respond to PEG 6000-associated drought stress imposed under *in vitro* conditions. However, this work aimed to investigate the effects of water deficiency stress generated by PEG 6000 on *in vitro* grown *V. phaleanosis* and evaluate how much polyethylene glycol is efficient in water deficit treatment for orchid plants.

The plant's chlorophyll content and photosynthetic rate were both reduced as a result of PEG-induced osmotic stress. Plant growth is hampered by water deficit, diminishing photosynthetic rate, and hindering metabolic processes. (Lawlor & Cornic, 2002). Water-stressed plants have lower biomass, chlorophyll content, and relative water content. (Specht et al., 2001).

Orchids was selected for microporpagatio due to its rare its high market demand and very little data is present for its invitro production.

Orchids require a lot of water and a high humidity level of 70 to 80 percent. In water-stressed conditions, PEG will aid the growth of cultured plants. Orchid growth will require even less water in the future (Yam et al., 2009). Commercial farmers used this strategy to cultivate unusual species and hybrids that were difficult to come by. (Singh &

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Kumar, 2009).

MATERIALS AND METHODS

Sample Collection

Explants samples of *V. phalaenopsis* were collected from Thailand, Bangkok for tissue culture. Explants were surface sterilized in 70% ethanol for 60 seconds, then immersed in a 0.1 percent aqueous mercuric chloride (HgCl2) solution for 25 minutes and washed five times in sterile distilled water.

Media Preparation

An initiation media was prepared. The basal media used was Murashige & Skoog (MS) medium. The basal Murashige and Skoog (1962) medium was supplemented with various growth regulators for shoot proliferation, callusing, and further regeneration of Orchid. Growth regulators and major salts were prepared in distilled water. The composition of media is given in appendix 1.All the medium components should be dissolved one at a time to distilled water. Finally, phytagel is added, and the solution is warmed till phytagel is dissolved. Maltose was added as a carbohydrates source. pH of the medium was adjusted at 5.77 sets the desired pH with NaOH or HCl. The 25 ml media was poured in jars and the autoclave at °C, 15 psi. For 15 minutes. The culture media is stored in the dark, dry, and clean cabinet of the culture room. We gave Different conditions of sterilization to in vitro sources of explants.

Table1: The Concentration of Media Ingredients

MEDIA NAME	HORMONE	HORMONE conc.	PEG CONC. gm/l
MS N	BAP	2 ml	Nil
MS P	NAA	1 ml	0.5gm

Water Deficit Treatment

Water potential was lowered by adding various amounts of polyethylene glycol (PEG) (molecular weight 8000; Sigma, St Louis, MO) to the growth medium. Approximately 0.5 gm of PEG solution was poured on top of an equal volume media put on the magnetic stirrer. After stirring it to add phytagel. Autoclave the media. After one week, checks the media for fungal or bacterial growth. The PEG solution had the same nutrient composition as the growth medium; nutrient solution without PEG was poured on the jars for the 'well-watered control medium, which gave a water potential for the phytagel

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medium. Water potentials were measured every 3 days by measuring the leaves, shoots, and callus of media with peg and media without PEG. The data are reported as the mean of the measured potentials.

Initiation

The media and surface area were cleaned with 70% ethanol using cotton. Before initiation, the leaf was washed twice with autoclaved distilled water to remove traces of bleach or any other contaminant. The dried & spoiled plant parts were removed. Orchid plants were then cultured in both MS N & MS P medium. The cultured jars were stored having photoperiod 16:8 (light: dark), 2000 lux light to optimize conditions. The initiation jars were inspected daily. The cultured pot showed results after15 days.

The media used had additional ingredients besides essential MS (see appendix 1). These other ingredients, along with the media, are mentioned in table no.1.

Each time one-liter media prepared was divided into two equal proportions to set two different formulations:

- 500 ml MS N (media without PEG) poured in jars.
- 500 ml media with PEG poured in jars.

This scheme turned the number of experiments twice, so 2 different experiments that were set finally are as follows:

1. MS + BAP +0.25gm½litre phytagel
2. MS + BAP + phytagel 0.25gm½litre +PEG

The culturing was done as described in methodology and observed for results.

RESULTS

Water deficits had a substantial impact on plant growth and metabolism. When PEG increased the water deficit in orchids, the leaves, callus, shoots, and roots growth and development slowed significantly compared to the relative control group.

Effects of Callus Growth in Response of PEG and Normal Medium in Orchid Plant

Table 2. Growth of Callus in Response to PEG and Normal Medium

Media CODES	MEDIA CONC	%age OF CALLUS INDUCTION	PROLIFERATION
OMS1	MS+NAA+BAP+EDTA	45%	++

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OMS2	MS+NAA+BAP+EDTA	41%	++
OMS3	MS+NAA+BAP+EDTA	22%	+
OMS4	MS+NAA+BAP+EDTA	43%	++
OMS5	MS+NAA+BAP+EDTA	20%	+
OMS6	MS+NAA+BAP+EDTA	33%	++
OMSP1	MS+NAA+BAP+EDTA+PEG	80%	++++
OMSP2	MS+NAA+BAP+EDTA+PEG	88%	++++
OMSP3	MS+NAA+BAP+EDTA+PEG	85%	++++
OMSP4	MS+NAA+BAP+EDTA+PEG	90%	++++
OMSP5	MS+NAA+BAP+EDTA+PEG	91%	++++
OMSP6	MS+NAA+BAP+EDTA+PEG	85%	++++

OMS= orchid media, OMSP =orchid media with PEG, +=poor, ++= fair, +++=good, ++++= excellent

The results represented by figure:1 prove that the MS N (MS Normal medium) did not promote the callus induction as it was promoted in the MS P (MS PEG medium). It proves that the PEG is the best chemical that can promote the growth of the orchid plant *(v.phalaenopsis)* in water-stressed conditions. Maximum callus induction was about 91% in MS P medium. This medium greatly affects the growth of callus by reducing the growth of leaves. It was almost half in the MS N medium, proved 45% callus induction in the results.

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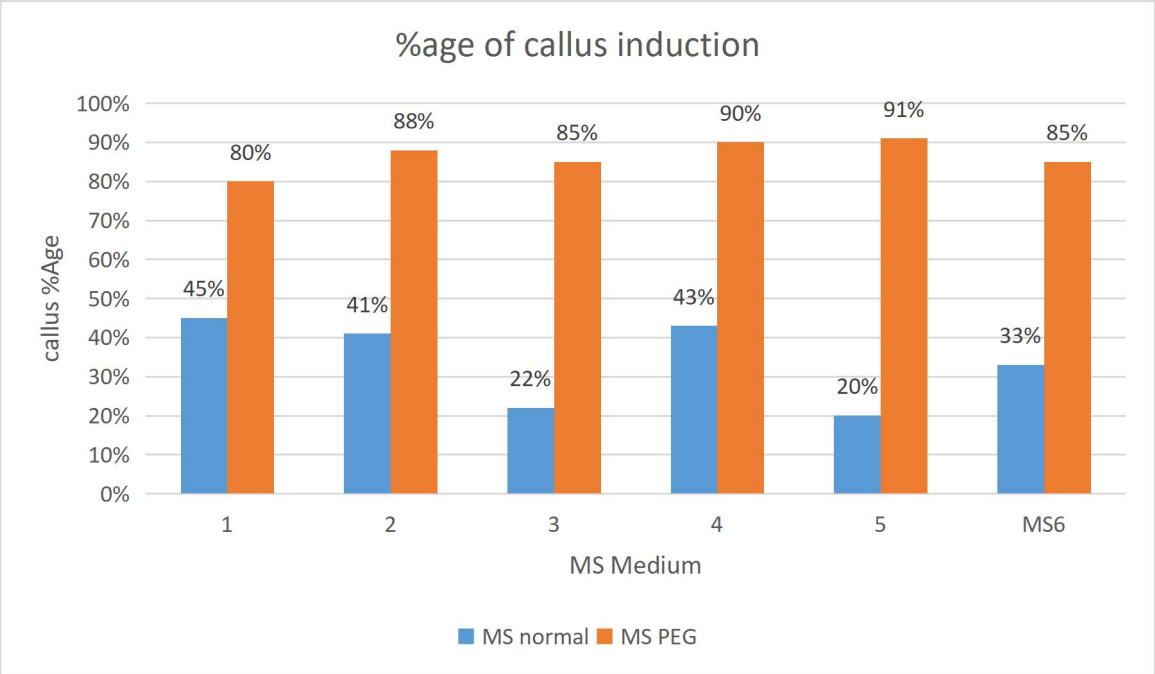

Figure: 1 Shows the % age of Callus Induction

The figure represents maximum height of the shoot. It compares the MS medium on the x-axis and the Max height of shoots on the y –axis. It is clear from the graphs MS normal medium promotes the growth of shoots but this is not the case in the MS medium with PEG, because PEG is known to reduce the growth of shoots to increase the life span of plant in water-stressed conditions. Bar 2 shows the jar code with a normal medium which has got a Maximum height of 3.3 cm of the shoot.

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Effects of Peg & Normal Medium in Orchid Plant on Shoots Induction, Maximum Height of Shoots & Number of Shoots

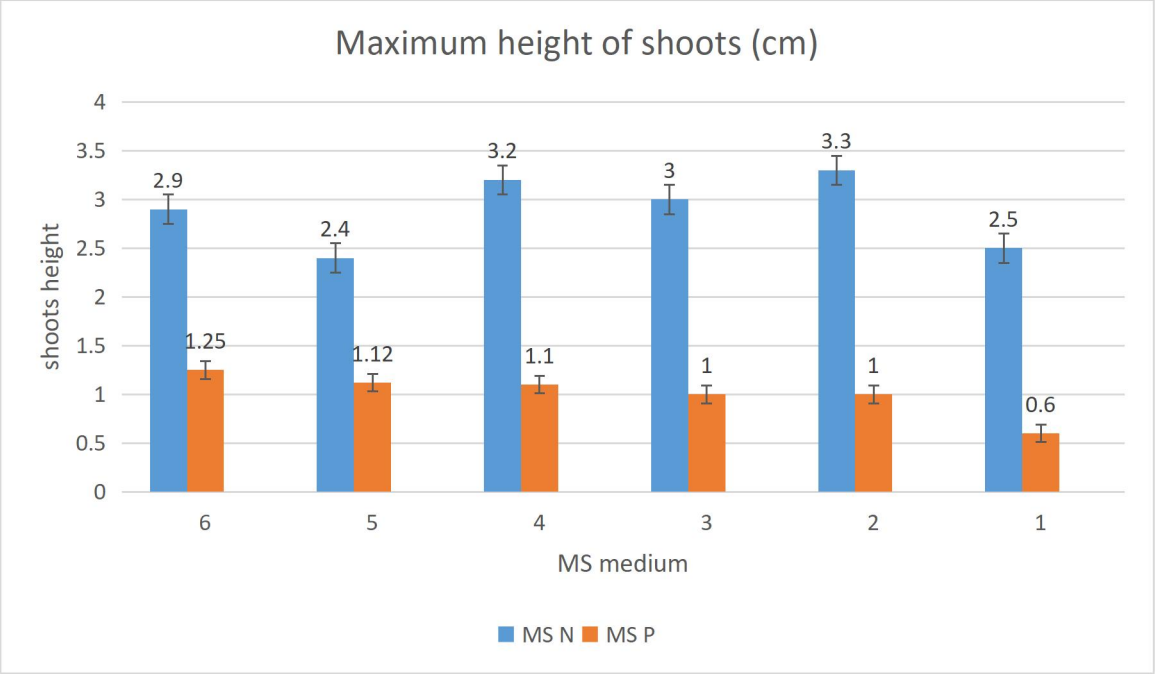

Figure: 2 Represent the MAX Height of Shoots in MS N & MS P Medium

MS normal medium appears to be more effective for the shoot induction in the orchid plant. More than 64% of the shoot induction is found in the MS N medium. MS PEG has suppressed the %age of the shoot induction as the results proved it to be 21% to bring the changes in the plant so that it could be able to survive its drought conditions by reducing the transpiration rates.

Table 3. Represents the NO. of Shoot Induction & MAX Height of Shoots on Orchid in MS Medium with PEG & without PEG

Jar codes	Media conc.	Max height of shoots MEAN±SEM cm	No of shoots MEAN±SEM	%age of shoot induction
OMS1	MS+NAA+BAP+EDTA	2.5±1.4	29.3±5.8	58.6%
OMS2	MS+NAA+BAP+EDTA	3.3±1.19	32±6.01	64%
OMS3	MS+NAA+BAP+EDTA	3±1.4	31.25±5.6	62%
OMS4	MS+NAA+BAP+EDTA	3.2±1.6	17.8±5.315	35%

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OMS5	MS+NAA+BAP+EDTA	2.4±1.4	24.76±7.9	49%
OMS6	MS+NAA+BAP+EDTA	2.9±1.5	24±6.7	48%
OMSP1	MS+NAA+BAP+EDTA+PEG	0.6±0.4	10.5±0.56	21%
OMSP2	MS+NAA+BAP+EDTA+PEG	1±0.8	8.5±0.57	17%
OMSP3	MS+NAA+BAP+EDTA+PEG	1±0.6	5.5±0.57	11%
OMSP4	MS+NAA+BAP+EDTA+PEG	1.1±0.29	7.3±1.5	14%
OMSP5	MS+NAA+BAP+EDTA+PEG	1.12±0.66	11.5±0.577	23%
OMSP6	MS+NAA+BAP+EDTA+PEG	1.25±0.71	6.5±0.577	13%

OMS= orchid media, OMSP =orchid media with PEG

Values are mean ± S.E.M;

S.E.M =Standard Error of Mean

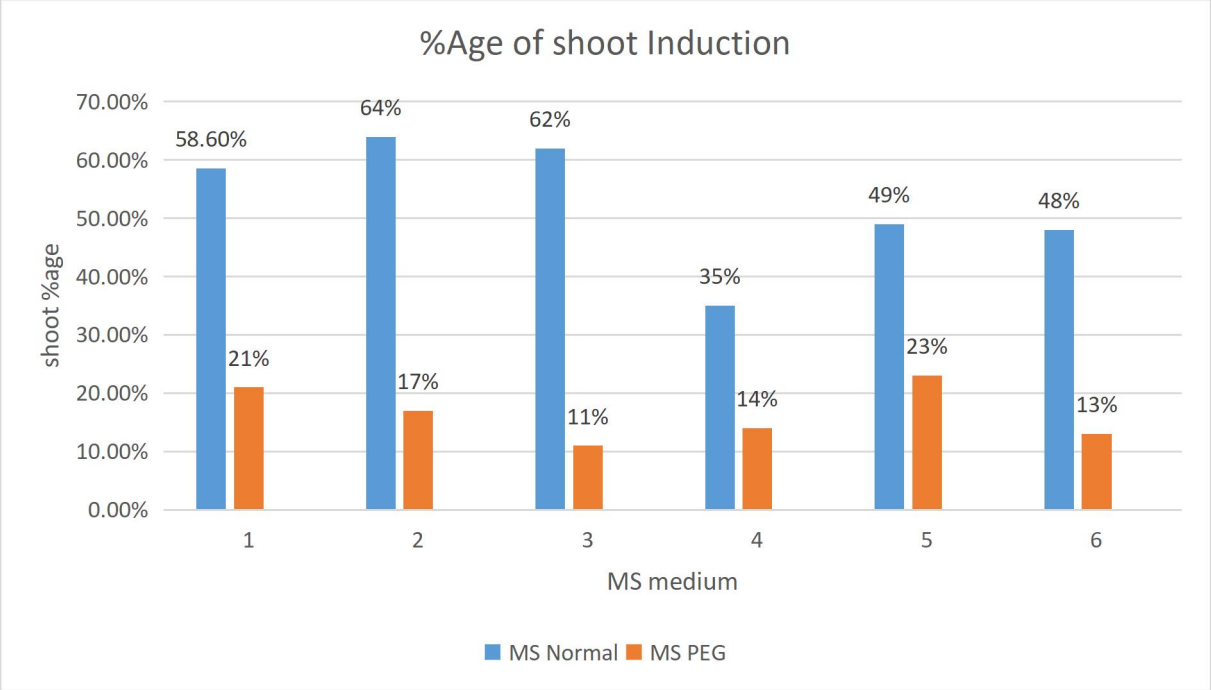

Figure: 2 Represents the Comparison b/w the %age of Shoot Inductions of MS normal Medium & MS PEG Medium

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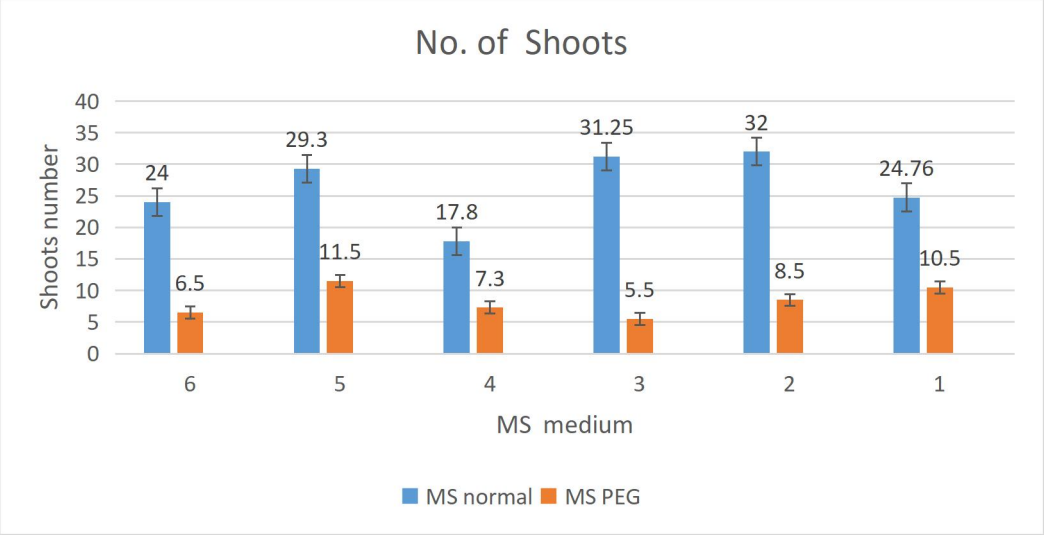

Figure: 3 Represents the Comparison b/w Number of Shoots in MS Normal Medium & MS PEG Medium

In the figure 3, the MS normal medium is found to be significantly more favorable for the growth of the shoots as the number of the shoots is 32 cm. On the other hand, MS PEG medium is not promoting the growth of shoots as the maximum number of shoots is only 11. As it was explained before that PEG reduces the growth of shoots for the growth of plants in less water.

Influence of Drought Stress of Leaf Induction

Leaf inductions were also suppressed by the MS P medium due to PEG as the best result of leaf induction was only 57%. And MS N medium promotes the leaf induction as the best result was 86%. As MS P medium was induced for the water-stressed condition that's why the impact must be on the leaves & stomata to reduce the transpiration process.

Table 4. Represents the % Age of Leaf Induction & Average No. of Leaves in MS N & MS P Medium

Jar codes	Media conc.	No of leaves MEAN±SEM	% Age of Leaf Induction	Proliferation
OMS1	MS+NAA+BAP+EDTA	39.5±11.733	79%	++++
OMS2	MS+NAA+BAP+EDTA	32.75±12.69	65.5%	+++
OMS3	MS+NAA+BAP+EDTA	33±2.34	66%	+++
OMS4	MS+NAA+BAP+EDTA	30.4±3.3	60.8%	+++

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OMS5	MS+NAA+BAP+EDTA	43.8±8.47	86%	++++
OMS6	MS+NAA+BAP+EDTA	39.8±9.21	80%	++++
OMSP1	MS+NAA+BAP+EDTA+PEG	25±1.74	50%	++
OMSP2	MS+NAA+BAP+EDTA+PEG	28.8±1.78	57.6%	++
OMSP3	MS+NAA+BAP+EDTA+PEG	23±2.55	46%	++
OMSP4	MS+NAA+BAP+EDTA+PEG	20.4±1.52	41%	++
OMSP5	MS+NAA+BAP+EDTA+PEG	24.75±14.863	49%	++
OMSP6	MS+NAA+BAP+EDTA+PEG	21.0±8.832	42%	+

OMS= orchid media, OMSP =orchid media with PEG

Values are mean ± S.E.M

S.E.M =Standard Error of Mean

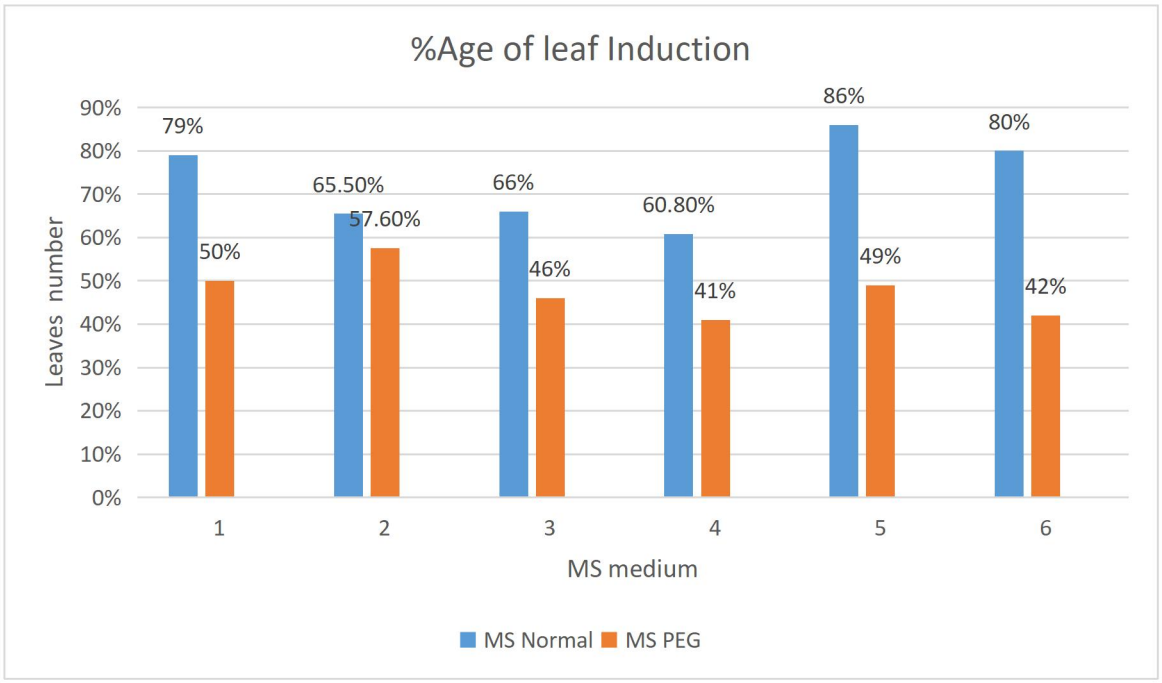

Figure: 4 represent the Comparisons of Leaf Induction in the MS N & MS P Medium

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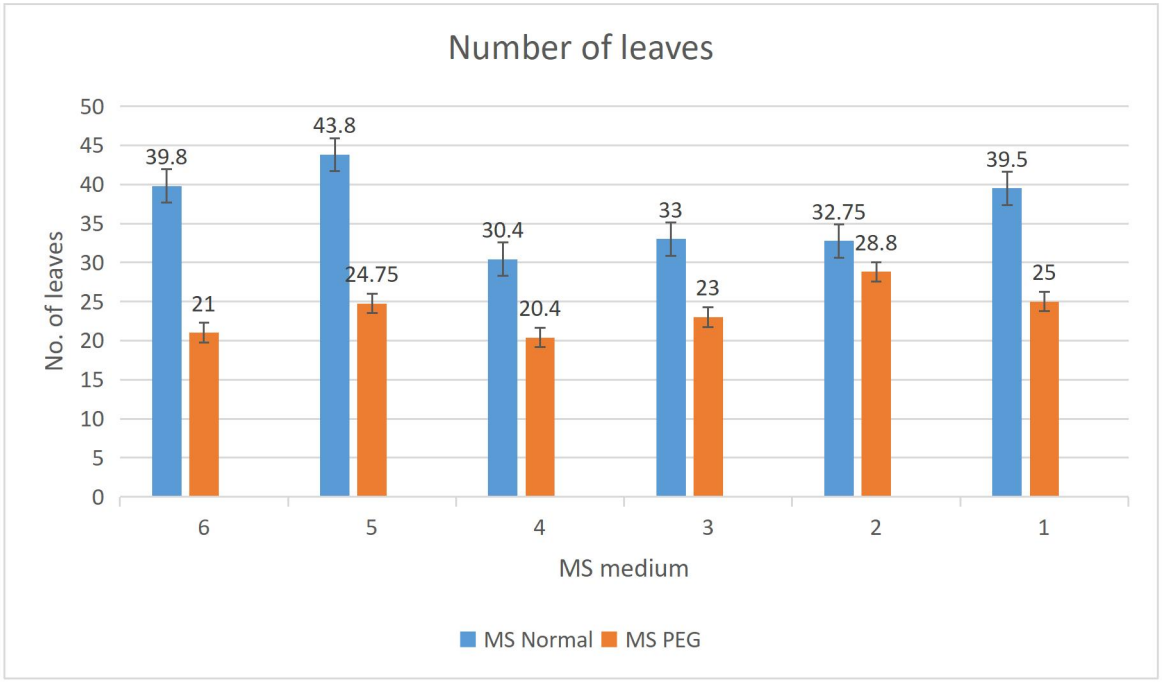

Figure 5. Represents a Comparison of the No. of Leaves of the MS P & MS N Medium

MS N=MS Normal, MS P=MS PEG

The figure shows the number of leaves was also reduced in the PEG medium as the number of leaves was only 23.8, and on the other hand, they are found to have fully grown in the MS normal medium as the number of leaves was 43.8. The effect of hormones like auxins, cytokinins, etc is fully expressed in the MS N medium.

DISCUSSION

Plant tolerance was heavily dependent on how plants adapt and respond physiologically and morphologically to many types of stress, particularly drought. Plants responded differently to water stress based on the intensity of the stress, the type of plant, and the age of the plant. On the other hand, water stress had a general effect on the size of plant organs, one of which was the narrowing and contraction of leaves. (Nadir et al., 2019)

In vitro drought stress using PEG significantly reduces the morphology. The current study compares the effect of the normal medium with the PEG medium on the growth of leaves, shoots, and calluses of orchid plants. The creation of drought conditions is not a problem since non-penetrating and inert polyethylene glycol (PEG), with its high molecular weight, induces water stress in plants (Rai et al. 2011). According to the findings, PEG 6000 causes osmotic stress, which significantly impacts a shoot, callus, and

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leaves. The Shoot growth was decreased in our results and was following Anwer et al., (Anwer et al., 2004). This study revealed leaf shrinkage, suppressed growth of leaves, and reduction in shoot length with the PEG medium. Piwowarczyk et al., in 2014 in their studies evaluated several physiological responses of PEG-generated osmotic stress and observed such as leaf shrinking, yellowing of root tips, and leaf curling after 7 days. (Piwowarczyk et al., 2014)

In the current study, we have revealed the shoots under treatment with PEG were reduced. The leaf induction was also reduced, so was the chlorophyll content. Our results correlate with the study of Shirazi et al., 2019 documented the effects of PEG-induced water stress on rice as reduction of shoot length and chlorophyll content of rice. (Shirazi et al., 2019). Similar work was also elaborated by Ebrahim et al, on bananas in which PEG worked to reduce shoot. (Ebrahim et al., 2006). Our results are consistent with the study of Ahmad et al., (2020b) on *Stevia rebaudiana* in which all shoot, root, and leaves are reduced. Our results were also consistent with the study of Nadir et al., 2019, as they also proved the plant to adapt the drought tolerance when treated with PEG. (Nadir et al., 2019).

CONCLUSION

Drought is a major constraint to agriculture production worldwide, as fertile lands are continuously rendered uncultivable due to drought stress. The present investigation is an attempt to survive orchid species to adapt to the tolerable drought environment. The current study showed that orchid species' growth and yield attributes (vappodes *phalaenopsis)* were significantly affected by PEG-induced drought stress. Different growth parameters including the percentage of explants shooting, mean shoots Plants length, the mean number of leaves, and the mean number of callus growth. After using PEG, the results further confirmed that Under PEG induces artificial stress situation, growth of orchid species was retarded concerning root, shoot growth and stay green. The ratio of callus induction percentage in MS N and MS P medium is 45:91, The ratio of leaf induction percentage in MS N and MS P medium is 86:57, the ratio of several leaves in MS N and MS P medium is 43:28**,** the ratio of shoot height in MS N and MS P medium was 3.3:1.25. These results are significant proof of the success of this experiment and for approval of PEG as a tolerable drought chemical.

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