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In vitro propagation of *Zamoculcas zamipholia* LODD., through indirect regeneration

Mahdi Alizadeh*, Azim Ghasemnejad, Mostafa Khoshal Sarmast, Yeganeh Basiri, Arezoo Jondou Aghleboub

Horticulture Department, Gorgan University of Agricultural Sciences & Natural Resources, Gorgan, Iran
**Email: mahdializadeh@gau.ac.ir*

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ABSTRACT

The Zamoculcas zamifolia LODD., commonly known as ZZ Plant, is considered as an ornamental foliage plant that its growing is recently getting into consideration in Iran. This plant is a virtuous choice for indoor use, due to its wonderful performance even without taking special care. The plant is naturally slow grown species and its propagation through conventional methods is also sluggish. So, due to these reasons, ZZ plant has got an expensive price in the market. Application of modern techniques such as micropropagation may provide its mass multiplication in shorter period as compared to conventional methods. In the present research work, the feasibility of in vitro propagation of this plant was evaluated. This experiment was undertaken as completely randomized design. The leaf segments procured from a healthy mother plant were surface sterilized with Colorax (Sodium Hypochlorite) and $HgCl_2$ and inoculated under in vitro conditions. The results showed that leaf explants of Zamoculcas may be efficiently established on MS medium comprising BA (2.0 mg/l) and NAA (0.2 mg/l) and produce callus tissues. The calli mass may produce maximum number of shoots (average 4 per each vessel) whenever sub-cultured on MS medium supplemented with BA (2 mg/l). The number of shoots was decreased if the IBA concentration was beyond 2 mg/l. Application of TDZ to regeneration medium had less number of microshoots as compared to BA treatment, but there was no significant statistical difference between these two treatments (P=0.05). Furthermore, the quality of plantlets induced by TDZ was higher than other treatments. The half strength MS medium supplemented with IBA (2 mg/l) and NAA (mg/l) is suggested for in vitro rooting of Zamoculcas microshoots. Application of NAA is also induced rooting, but the effectiveness was less than IBA.

***Keywords*:** Callus, differentiation**,** proliferation, propagation, ZZ plant

INTRODUCTION

Zamioculcas zamiifolia (Lodd.), also known as ZZ plant, is a monocot, tropical ornamental plant belongs to Araceae and native to Eastern Africa. The *Z. zamiifolia* is frequently used medicinally in the African countries. The juice from the leaves is used medicinally to treat earache and inflammatory conditions. Roots are used as a local application to treat ulceration by the Sukuma people in northwestern Tanzania (Moullec *et al*., 2015). The ZZ plant is enjoyed for its unique appearance, its ability to grow under low light conditions, and its tolerance to drought. The ZZ's naturally glossy leaves are so shiny that the plant appears to have been polished (Alizadeh *et al*., 2019). It produces succulent rhizomes at the base of its attractive dark green and glossy foliage (Lopez *et al*., 2009). It has recently gained commercial ornamental importance (Seneviratne *et al*., 2020) and the plants with green, dark purple and variegated green colors are presently available (Mayers, 2024). The dark purple is known as Black Zamiifolia, has more fan in Iran. Furthermore, plants with dwarf feature were recently produced through mutation induced by colchicine (Seneviratne *et al.,* 2020).

Zamiifolia, is a popular and low-maintenance houseplant. It can tolerate low light conditions, but prefers bright, indirect light. Direct sunlight should be avoided as it can scorch the leaves. The plant also grows well under fluorescent lighting and that's why it has gained popularity as a recent addition to pot plants especially in Iranian apartments and official areas. Overwatering can lead to root rot. a

well-draining potting soil should be used and a mix of regular potting soil with some perlite or sand works well. They can tolerate dry air and prefer average to warm temperatures and normal indoor humidity levels (Bown, 2000; Henny and Chen, 2013).

Propagation of Zamiifolia can be readily undertaken through division or leaf cuttings (Lopez *et al*., 2009; Malla *et al*., 2023). When re-potting a mature ZZ plant, developed rhizomes (underground stems) are carefully removed and gently separated the rhizomes into sections, ensuring that each section has both roots and stems. Each divided section is planted in its own pot with well-draining soil. The pots are placed in a warm, bright location but indirect sunlight. New growth would emerge from the divided sections within a few weeks.

The method of propagation through leaf cutting is more common especially for commercial mass multiplication and also when there are restrictions in terms of plant materials (Badizadegan *et al*., 2023). The mature leaves are grown individually or in groups in pots with a light substrate (Chen and Stump, 2006). The substrate should be moistened and the pots should be placed in a shady place (light intensity of 1000 to 1500 foot candles is enough). The rooting substrate can be a mixture of 60% peat and 40% perlite. This substrate should have favourable physical properties and its pH should be between 6 and 7 and the electrical conductivity of its saturated extract should be less than 2 ds/m (Chen *et al.*, 2004). Review of the literature with respect to the type of Zamophilia leaf cutting (Blanchard and Lopez, 2007), revealed that the leaf can be cut in different ways (Fig.1). The whole intact leaflet may be planted (a), or each leaflet may be cut from the middle part and each piece is inserted as an individual cutting. The b and c represent the upper and lower part of the leaflet, respectively). The study noted that both the upper and lower parts of the leaflet were capable of rooting successfully. However, the researchers observed a unique phenomenon when the end piece of the leaf was planted (Fig. 1b). This method resulted in the induction of more rhizomes, leading to the formation of additional stems. Consequently, this particular cutting technique was found to be more effective in stimulating rhizome growth and ultimately producing more stems.

Both stem division and leaf cutting techniques are extensively common in greenhouses and nurseries (Badizadegan *et al.*, 2023). However, the slow growth habit of ZZ plant (Henny and Chen, 2013) is a drawback for its mass propagation. Tissue culture techniques provide an opportunity for the large-scale production of an elite material and plants of commercial interest (Alizadeh *et al*., 2023).The micropropagation protocols for some Araceae genera such as *Alocasia*, *Anthurium*, *Aglaonema*, *Dieffenbachia*, *Philodendron*, *Spathiphyllum* and *Syngonium* has been already reported (Chen *et al*., 2006; Stanly *et al*., 2012; Chen *et al.*, 2018; Chen *et al*., 2012). Also, there are some contributions for *in vitro* propagation of green leaved Zamiifolia. The researchers studied the role of basal medium, plant growth regulators and also types of explants. Heping and Peng (2003) developed plantlets from leaf explants. Papafotiou and Martini (2009) demonstrated the effect of the inoculation position of leaf explants concerning growth regulators during the micropropagation of ZZ plant. A complete micropropagation protocol for Black-Leaved *Zamioculcas zamiifolia* was recently published by Pourhassan *et al*. (2023). In the present research work, in a series of experiments the feasibility of *in vitro* propagation of *Z. zamifolia* was evaluated.

MATERIALS AND METHODS

The present research was carried out in the Plant Tissue Culture Laboratory, located in the Department of Horticultural Sciences, Faculty of Plant Production, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. The greenhouse grown, mother and healthy plant of Zamifolia (*Z. zamifolia* LODD.) was obtained from Hirkan Plant Tissue Culture Company, Gorgan. The young leaves of the shoot tip and the base of the branch were removed, and the leaves in the middle part of the stem were used to prepare the explants.

Plant growth regulators benzyladenine BA, naphthalene-acetic acid NAA and thidiazuron TDZ were used. The stock solution of these substances was prepared with a concentration of 1 mg/ml (Alizadeh *et al*., 2019) and the required amount was added to the desired medium during the preparation of the culture medium. The types and concentrations of growth regulators for different stages of micropropagation are shown in Table 1.

A previous tissue culture study (Anonymous, 2014) had already documented a report on Zamophilia plant. Therefore, the MS medium supplemented with BA (2 mg/l) and NAA (0.2 mg/l) was chosen for callus induction, as it was recommended in the aforementioned report. The leaf explants were then inoculated on this medium using two surface sterilization methods.The leaf samples were firstly pre-treated with fungicide (Carbendazim 2 g/l for 3 hours). Then, these were transferred to a Laminar Air Flow hood where, sterilization procedures were followed. Alcohol, Colorax and mercury chloride were used for surface sterilization. Sterilization was done by two methods, which are briefly called "Colorax method" and "Mercuric method". The details of surface sterilization methods were as following:

Colorax method : 70% alcohol for 30 seconds, several rinses with sterile water, Colorax 15% for 20 minutes, several rinses with sterile water, Colorax 10% for 10 minutes, several rinses with sterile water, and then inoculation were done individually in each container.

Mercuric method : 70% alcohol for 30 seconds, several times of rinsing with sterile water, 0.1% mercury chloride for 5 minutes, several times of rinsing with sterile water and then inoculation were done individually in each container.

The degree of contamination, the percentage of establishment and the degree of browning or burning of the samples after the inoculation of leaf pieces were evaluated to determine the effectiveness of the disinfection method.

Following the callus induction in the leaf explants, the callus pieces were sub-cultured several times on the same culture medium or on the hormone-free medium. Therefore, several growing cultures containing healthy and actively growing callus were produced. The callus mass was then sub-cultured on different regeneration medium (Table 1). As much as possible, it was tried that the size of all callus pieces were the same and at least "pea size" when sub-cultured. After 6 weeks, data were taken from the regenerated samples and their average was used for statistical analysis.

The regenerated shoots in the previous section were inoculated in $^{1}/_{2}$ MS medium to induce rooting. Here, the entire mass of callus and its regenerated branches were removed from the culture at once. The branches were cut from the junction with the callus with a scalpel and inoculated individually on the rooting medium.

In the stages of regeneration and rooting, the number of produced branches was recorded and their length was measured. The callus volume along with the branches and the number of leaves were recorded. Callus growth rate was determined by observation and scoring as (0) no growth; (1) 30-50% volume increase in the callus; (2) 50-100% and (3)100% or more increase in callus volume.

The data collected from this experiment was analyzed using a completely randomized design with four replications. Mean data comparison was conducted using the LSD test at a 5% probability level with the assistance of SAS statistical software.

RESULTS AND DISCUSSION

In the present study, the possibility of regenerating whole Zamophilia plants from leaf explants was evaluated. Leaf pieces were cultured on commercial MS culture medium. After callus production, the callus pieces were sub-cultured on regeneration culture medium. The regenerated branches were then transferred to the rooting medium. The results of each of these steps are reported hereafter.

Culture establishment

In both methods of sterilization, some samples were successfully sterilized and established on the culture medium, but in general, Mercuric method was more effective. The degree of browning or tissue burning was almost the same in both methods. In both methods, a number of Zamophilia leaf pieces were successfully established in the jam bottles and callus formation was observed about 4 to 5 weeks after inoculation. The amount of culture establishment in these two methods has a significant difference ($P = 0.05$) and shows the better efficiency of mercury chloride (Fig. 2). What is certain is that mercuric chloride is dangerous for the environment as well as plant tissue culture user (Alizadeh, 2010). Therefore, the observations showed that using Colorax can also successfully establish enough *in vitro* samples in this plant (Fig. 2).

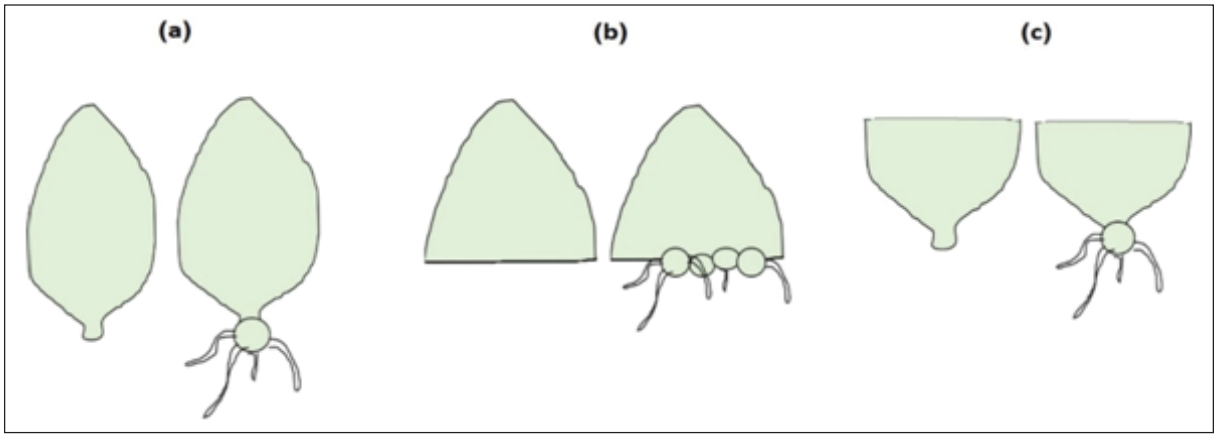

Fig. 1. Types of leaf cuttings in *Zamoculcaszamipholia*. Complete intact leaf (a), upper part of the leaf (b), lower part of the leaf (c). When the leaf iss cut and its upper part is planted, the number of induced rhizomes will be more (Drawing by author- M. Alizadeh).

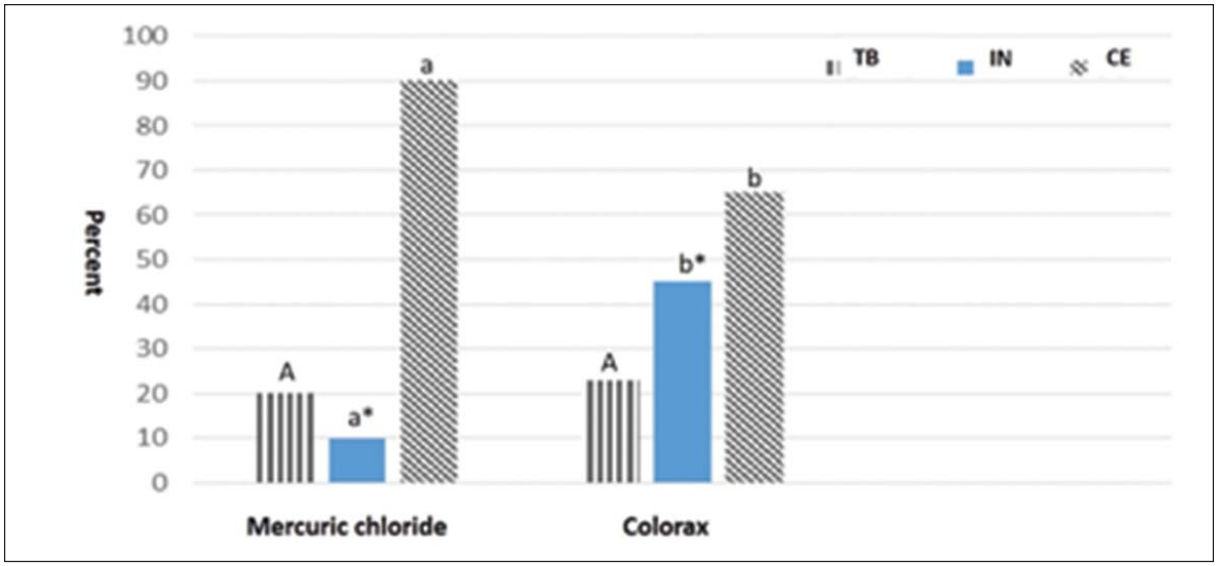

Fig. 2. The efficacy of two sterilization methods for *in vitro* culture establishment of ZZ plant. TB, IN and CE represent tissue browning, infection and culture establishment, respectively.

Shoot regeneration and proliferation

According to the recorded data, the leaf explants of ZZ plant were usually induced to produce callus tissue 4 to 5 weeks after inoculation. In the third week, the leaf parts were swollen and some samples were bent up from the middle due to the enlargement and were separated from the culture medium. From the third week onwards, callus formation appearedas tiny, snow-like particles on the cut edges of the explant tissue. It should be noted that, unlike its leaves, which are very shiny and green, the callus of ZZ plant is absolutely dark brown to black in color (Fig. 3). Regeneration from callus tissue is difficult in some plants (George, 1993), but according to the results of the present study, in the ZZ plant, just BA supplementation to the culture medium is sufficient to encourage the callus tissue and differentiation into shoot bud. A fascinating observation made in this section is the spontaneous branching of callus in certain culture containers when left unattended or uncultured for a period. As the water content decreases in the environment, the callus unexpectedly initiates branching. This phenomenon suggests that stress on callus tissue cells plays a crucial role, prompting plant cells to regenerate in order to ensure their survival (Alizadeh *et al.*, 2019).

Fig. 3. Different stages of *in vitro* propagation of ZZ plant. Inoculation of leaf explant (Up, left); shoot regeneration from callus tissue (Up, right); The influence of BA on shoot regeneration (Bottom, left); a whole rooted *Zamoculcas* plantlet (Bottom, right).

The number of regenerated branches and their length in different culture media was statistically different among treatments (Table 2). The difference in shoot regeneration from callus samples in response to different growth regulators was shown in Table 3. The highest number of shoots was observed in medium supplemented with BA (2.0 mg/l). It seems that increasing the concentration of cytokinin or adding NAA can lead to a decrease in the number of shoots. The TDZ also induced shoot proliferation, but its efficiency is not as high as BA. Based on the enquiry, it was found that the cost of TDZ is significantly greater than that of BA. As a result, when considering the commercial propagation of Zamophilia, it can be stated that the same concentration of BA at 2 mg/l is an effective and superior treatment option.

In other plants of Araceae family, such as *Anthurium* and *Pothos*, treatment with BA or another type of cytokinin such as kinetin has led to regeneration, which is consistent with the results of this research (Hamidah *et al.*, 1997; Zhang *et al*., 2005). The regenerated shoots in different culture media were not the same in terms of size (Table 2). Increasing the concentration of BA in the shoot proliferation medium causes the shoots to become shorter. Considering that this negative effect was also observed in the number of regenerated shoots, it is recommended not to use more than 2 mg/l BA in this plant. Also, the possibility of vitrification, which is an undesirable phenomenon, also exists in high concentrations of cytokinin (Alizadeh, 2010). Inclusion of NAA to the shoot proliferation medium caused the formation of a large volume of callus at the base of the shoots and the regenerated shoots were also less. In general, application of 2 mg/l BA for branching

Table 1: Culture medium and growth regulators treatments for *in vitro* propagation of ZZ plant

Plant growth regulator (mg/l)	Basal medium	Growth stage
Callus induction	MS	● BA (2.0) + NAA (0.1)
Shoot proliferation	MS	● BA (2.0)
		● BA (4.0)
		● BA (2.0) + NAA (0.2)
		● BA (4.0) +NAA (0.2)
		● TDZ (1.5)
Root induction	MS	● IBA (2.0)
		● IBA (2.0) + NAA (0.5)
		● NAA (0.5)

BA: Benzyl adenine; NAA: Naphtalene acetic acid; TDZ: Thidiazuron

Table 2: Variance analysis and mean data for *in vitro* traits of ZZ shoot proliferation stage

Sources of Variations	df	No. of Shoots	Shoot length	Callus volume	visual quality
Treatment	4	3.43*	1.11*	1.4 ns	0.56 ns
Error	10	1.4	1.15	1.6	1.66
* and ns, are significant at 5% level and no significant difference, respectively.					
		Mean comparison data			
BA (2.0)		3.67 a	50.3 a	1.1 a	1.6 a
BA (4.0)		1.43 b	34.6 ab	1.4 a	1.9 a
BA (2.0) + NAA (0.2)		2.59 ab	49.9 a	1.1 a	1.6 a
BA (4.0) +NAA (0.2)		1.10 b	18.2 b	1.6 a	1.3 a
TDZ (1.5)		2.10 ab	50.1 a	1.6 a	2.2 a

• The data followed by the same letter in each column do not have statistically difference.

Table 3: Variance analysis and mean data for *in vitro* traits of ZZ plant at rooting stage

Sources of Variations	df	No. of roots	Shoot length (mm)	No of leaves
Treatment	**3**	**26.98***	**0.93 ns**	**1.8 ns**
Error	**16**	**1.4**	**1.15**	**1.6**
* and ns, are significant at 5% level and no significant difference, respectively.				
		Mean comparison data		
$^1/_2$ MS free hormone (Control)		2.33 b	44.56 ns	2.34 ns
$^1/_2$ MS+ IBA (2.0)		2.99 a	43.65 ns	2.76 ns
$^1/_2$ MS + IBA (2.0) + NAA (0.5)		7.21 b	44.32 ns	2.41 ns
$^1/_2$ MS+ NAA (0.5)		2.45 b	44.28 ns	2.65 ns

* and ns are respectively significant at 5% level and no significant difference.

seems to be a reasonable combination. Application of TDZ (1.5 mg/l) in the proliferation medium produced fewer shoots than BA, but they were not statistically different from each other (Table 2).

Rooting

In this experiment IBA and NAA and their combined treatment was tested for rooting and the results obtained were as follows. Since the ZZ plant is a rhizomatous plant, no special problem was observed in its *in vitro* rooting. Therefore, even the shoots inoculated in $^{1}/_{2}$ MS medium without growth regulator also produced roots. Adding auxin significantly (Table 3) increased the number of roots, but the best result was obtained in the combination treatment of auxin. The number of roots produced in the treatment of IBA and NAA when they were used alone was not significantly different from the control, but while their combined application, more roots were observed (Table 3).

CONCLUSION

Based on the findings, it is recommended to use a MS medium comprising BA (2.0 mg/l) + NAA (0.2 mg/l) for efficient culture establishment of leaflet segments of ZZ plant and subsequent callus tissue production. Sub-culturing the calli mass on MS medium with BA (2 mg/l) is advised for maximizing the number of shoots. Further research could explore the potential for optimizing the use of TDZ to achieve a balance between microshoot production and plantlet quality, and investigate alternative methods for *in vitro* rooting that may enhance overall efficiency and yield.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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