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# IN VITRO DIPLOIDIZATION OF HAPLOID PLANTLET FROM ANTHER CULTURE OF EGGPLANT (*Solanum melongena* L.) USING PENDIMETHALIN

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**SUMMARY**

The availability of anther culture methods for producing pure lines in a doubled-haploid (DH) plant form makes it possible to accelerate the development of hybrid varieties in eggplant. Previous studies have developed an efficient eggplant anther culture method. However, the formation of spontaneous DH plants through this method is relatively low, ranging from 25%–30%. Recently several studies reported that pendimethalin effectively duplicates the chromosome numbers of several plant species in vitro. This study aimed to determine the pendimethalin effect on the diploidization of eggplant haploid plantlets from anther culture. The study compared three concentrations of antimitotic pendimethalin: 100 µM, 300 µM, and 1000 µM in three incubation durations: two, four, and six days. The results showed the treatment with a pendimethalin concentration of 300 µM incubated for two days gave the highest level of plantlet diploidization at 75.0%. In vitro, chromosomal duplication treatment using pendimethalin with different concentrations and duration of incubation time affected the plantlet survival and growth of eggplant haploid plantlets. The increase in pendimethalin concentration and incubation duration inhibited the physiology and growth of plantlets and affected alteration in the ploidy level of eggplant haploid plantlets.

**Keywords:** diploidization, doubled haploid, *Solanum melongena* L., pendimethalin

**Key findings:** Pendimethalin can effectively duplicate the chromosome numbers of haploid plantlets of eggplant in vitro.

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## INTRODUCTION

The development of eggplant hybrid varieties is highly dependent on the available selected pure lines as parents (Mir *et al.,* 2021). The availability of anther culture methods for producing pure lines in a doubled-haploid (DH) plant form makes it possible to accelerate the development of hybrid varieties in eggplant. The time required for producing DH plants through anther culture techniques is much faster than conventional methods through

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selfing and recurrent selection. Obtaining doubled haploids can occur only in one generation of offspring so that it can reduce the plant breeding cost and time (Rotino *et al.,* 1997; Caguiat and Hautea, 2014; Kumar *et al*., 2016). The DH plants produced can be directly evaluated and selected as new hybrid parents.

Spontaneous formation of DH plants can occur during the anther culture process, mainly due to the activity of cell fusion, endoreduplication, and endomitosis (Seguí-Simarro and Nuez, 2008). Previous studies reported the development of an efficient eggplant anther culture method (Chauhan *et al*., 2016; Mulyana, 2022). However, the formation of spontaneous DH plants through this method is relatively low, ranging from 25%–30%. Therefore, it is necessary to carry out the diploidization process of haploid plant chromosomes to obtain DH plants and restore their fertility for use in the breeding scheme. Başay *et al*. (2011) stated that the optimal method for DH plant production through anther culture gains support from the availability of optimal haploid chromosome diploidization methods.

Generally, chemical antimitotic agents, such as colchicine, are used for in vitro chromosome duplication in various plant species (Bürün and Emiroğlu, 2008; Solmaz *et al.,* 2011; Cola *et al.,* 2014; Mo *et al.,* 2020). Colchicine use as a chromosomal duplication agent can inhibit the alignment and separation of chromosomes during mitosis (Touchell *et al.,* 2020). However, colchicine as an anti-mitotic agent is not always applicable, whereas the diploidization process often occurs in a sectoral manner. In addition, colchicine is carcinogenic and needs skilled personnel for its effective handling. It is also relatively expensive and unaffordable for some laboratories.

Recently several studies reported that pendimethalin could effectively duplicate the chromosome numbers of several plant species in vitro (Suping *et al.,* 2016; Aslam *et al.,* 2017; Ren *et al.,* 2018; Jiang *et al.,* 2020). Generally, pendimethalin use helps control broadleaf weeds and annual grasses at pre-growth and post-growth stages (Anghel *et al.,* 2019). Pendimethalin is an herbicide belonging to the dinitroaniline class that can act as an anti-microtubular that inhibits chromosome separation and cell wall formation in mitosis (Promkaew *et al.,* 2010). This anti-microtubular activity is similar to colchicine's ability to cause a chromosomal doubling in plant cells. In addition, pendimethalin is much more economical than colchicine and can serve as its alternative. This study aimed to determine the effect of pendimethalin on the diploidization of eggplant haploid plantlets from anther culture.

## MATERIALS AND METHODS

### Plant material

The research took place from December 2021 to March 2022 at the Laboratory of the Cell Biology and Tissue Culture Research Group of ICABIOGRAD, Bogor, Indonesia. The plant material was haploid plantlets from anther culture obtained from previous studies (Mulyana, 2022).

### Experimental design

The experiment arrangement used a completely randomized factorial design of two experimental factors, i.e., the antimitotic agent pendimethalin added to the Murashige and Skoog (1962) medium (K) and the duration of chromosomal duplication induction (D). The antimitotic agent pendimethalin factor (K) included three concentrations: 100 µM (K1), 300 µM (K2), and 1000 µM (K3). The chromosomal duplication induction duration factor comprised of three levels: two days (D1), four days (D2), and six days (D3). Each treatment combination was replicated four times to obtain 36 experimental units. One experimental unit consisted of one bottle containing 10 haploid shoots. The haploid shoots were treated in the treatment medium under light conditions with a temperature of ±25 °C for the duration of each treatment. For the ploidy examination, a control treatment comprised of haploid shoots not treated with pendimethalin was used.

### Chromosomal duplication treatment

The performed chromosomal duplication in vitro followed the modified method of Ren *et al.* (2018). Culturing haploid plantlet shoots occurred on an MS0 medium containing the anti-mitotic agent pendimethalin with concentrations of 100 µM, 300 µM, or 1000 µM. Afterward, the shoots received incubation under light conditions for two, four, or six days. At the end of the duration treatment, transferring the shoots proceeded to a new medium with the formulation of B5 (Gamborg *et al*., 1968) + 0.5 mg/l IBA + 2% sucrose, pH 5.8 to stimulate new root growth (Hassanein *et al.,* 2020).

**Ploidy level analysis**

Plantlets to be checked for ploidy levels were fully rooted plants. Cell nuclei were extracted from in vitro young leaf tissues by employing the modified method of Doležel *et al.* (1989) and then stained with propidium iodide. The ploidy analysis was conducted using flow cytometry Guava EasyCyte 5 (Luminex, USA).

**Experimental observations**

Observations were conducted on the following: 1) the number of surviving explants and the percentage of surviving explants (the number of surviving explants divided by the number of explants treated multiplied by 100%); 2) the number of short stature plantlets and the percentage of short stature plantlets (the number of short stature plantlets divided by the number of explants treated and multiplied by 100%); 3) the number of plantlets with a certain ploidy level and the percentage of the number of plantlets with a certain ploidy level (the number of plantlets with a certain ploidy level [1×, 2×, 3×, 4×, or mixoploidy] divided by the number of plantlets checked for ploidy multiplied by 100%), and 4) the percentage of root formation (the number of rooted plantlets divided by the number of total treated plantlets multiplied by 100%). Observations and analysis of ploidy level by flow cytometry were carried out at 30 days after culture.

**Statistical analysis**

Statistical analysis used analysis of variance (ANOVA) and continued with Duncan's Multiple Range Test at a significant level of 5% if a substantial effect occurred. Data on ploidy observation were analyzed as a single-factor experiment, using Rstudio and MS Excel 2010 software.

**RESULTS**

**Effects of pendimethalin on plantlet survival and growth**

Figure 1 presents the different stages of in vitro diploidization of eggplant haploid plantlets using the pendimethalin. The results showed a significant effect of differences in pendimethalin concentrations and incubation duration on the growth of eggplant haploid plantlets. The results reveal the observation variables for the number and percentage of surviving explants and the observed variables for the number and percentage of short-stature plantlets (Figure 2). The interaction of pendimethalin concentration and incubation duration was not significant.

The highest number and percentage of surviving plantlets resulted from explants treated with the pendimethalin concentration of 100 µM and incubation duration of two days. Likewise, the highest number and percentage of short-stature plantlets resulted from in vitro shoots treated with the pendimethalin concentration of 1000 µM and incubation duration of six days.

The results showed no significant effect of differences in the pendimethalin concentration and incubation duration on the percentage of root formation in eggplant haploid plantlets (Figure 3A). Pendimethalin treatment with different concentrations and incubation duration did not affect root formation. Most of the shoot's explants used were still able to form roots after the treatment of pendimethalin (Figure 3B).

**Effect of pendimethalin treatment on chromosomal duplication**

Analysis of ploidy levels using flow cytometry results in the peak of histograms indicating differences in the ploidy level of regenerants (Figure 4). The average histogram peaks of haploid plantlet (1×) appeared around gate $10^3$ epifluorescence emitted by the cell nucleus, with the diploid/DH (2×), triploid (3×), and tetraploid (4×) plantlet histogram peaks appearing in multiples of 2, 3, and 4 times of the plantlet haploid (1×) gates.

The results showed a significant effect of differences in the pendimethalin concentration and incubation duration on the number and percentage of eggplant haploid plantlet chromosome duplications (Figure 5). The highest number and percent chromosomal duplications resulted from in vitro shoots treated with a pendimethalin concentration of 300 µM and an incubation duration of two days. The 300 µM pendimethalin treatment increased the number and percentage of chromosomal duplications. When amplified to 1000 µM pendimethalin treatment, these decreased. The number and percent of chromosomal duplications decreased as the duration of treatment increased. Both of these may have connections with the low number of explants that survived when treated with increasing concentrations of pendimethalin up to 1000 µM and incubation duration from two to six days, reducing the number and percentage of chromosome duplications. The

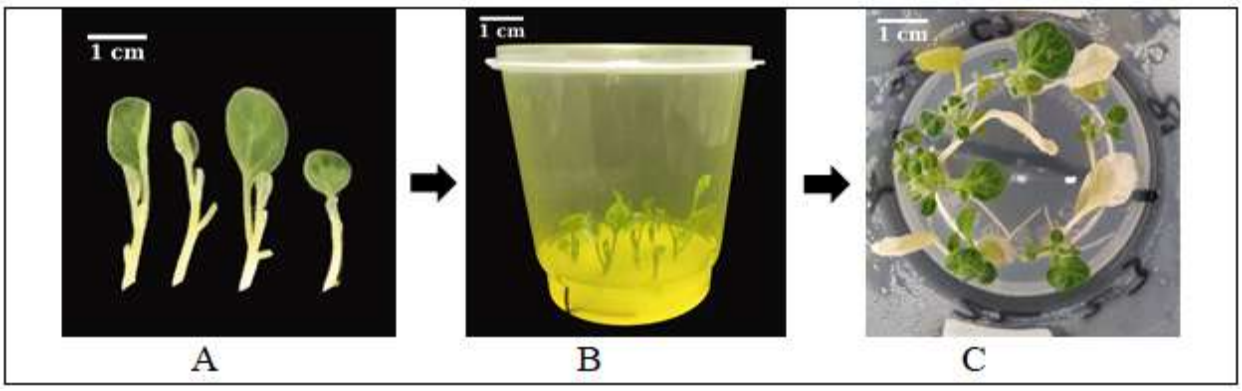


**Figure 1.** Stages of in vitro diploidization of eggplant haploid plantlets using pendimethalin. A) Healthy haploid shoot, B) Chromosomal duplication treatment using pendimethalin 300 µM for two days, and C) Rooted plantlets 21 days after transfer to fresh medium ready for ploidy checked.

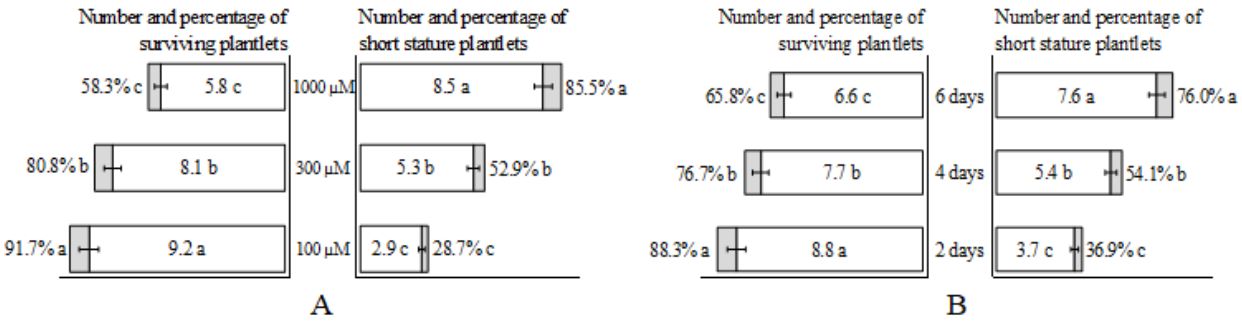


**Figure 2.** Number and percentage (%) of surviving explants and number and percentage (%) of short stature plantlets A) Based on pendimethalin concentration (µM) and B) duration of incubation treatment (days).

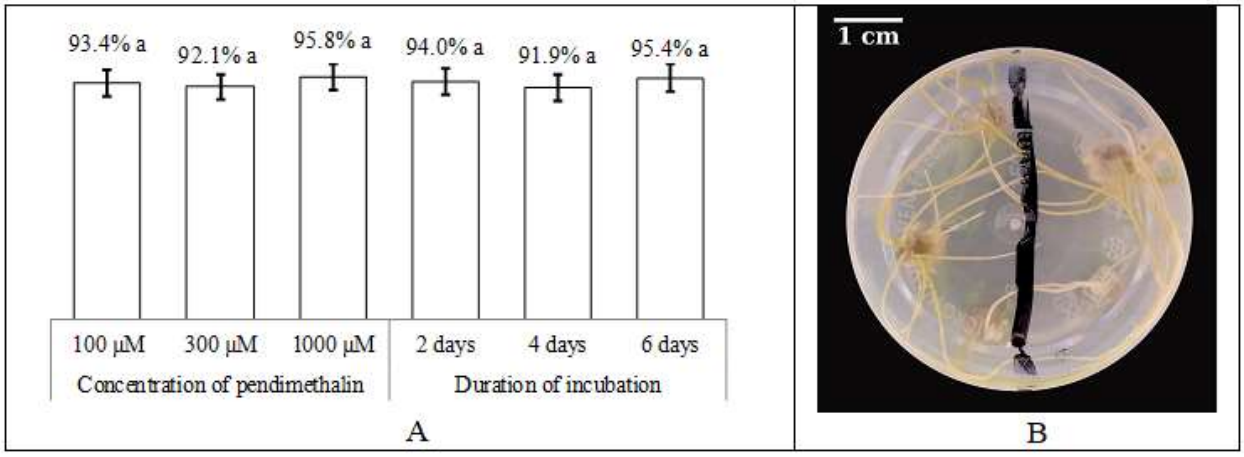


**Figure 3.** A) Percentage of root formation of eggplant haploid plantlet after chromosome duplication treatment (%) and B) Root formation of eggplant plantlets after chromosomal duplication treatment.

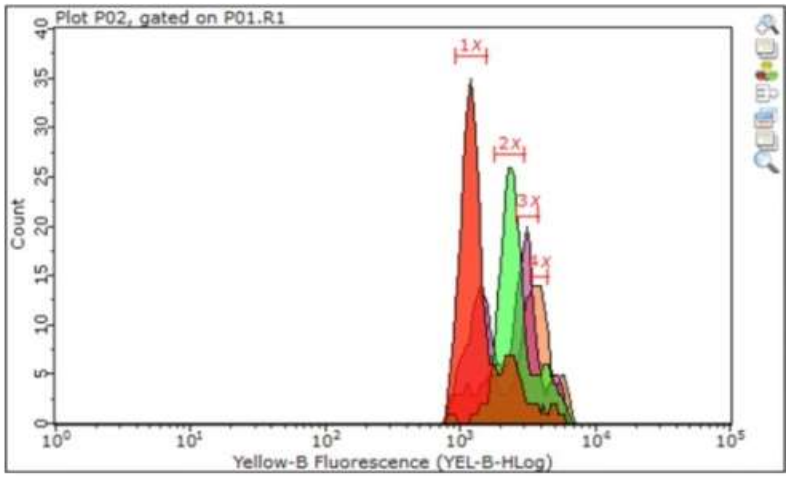


**Figure 4.** Overlay of histogram peaks resulted on the ploidy level analysis of eggplant using flow cytometry.

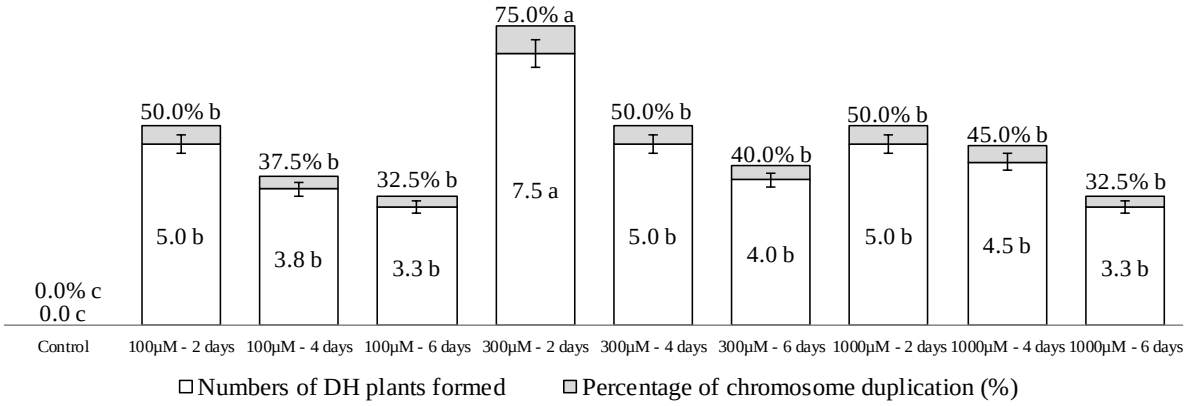


**Figure 5.** Average number and percentage (%) of chromosome duplication of eggplant haploid plantlet to doubled haploid form.

total number and percent of chromosomal duplications of haploid plantlets and plantlets with a certain ploidy level (1×, 2×, 3×, 4×, or mixoploidy) in this study appear in Table 1.

## DISCUSSION

Previous studies reported that increasing concentrations and incubation periods in media containing pendimethalin affected plantlet growth by inhibiting physiological processes (Ren *et al.*, 2018; Jiang *et al.*, 2020). Growth is the best variable to evaluate plants exposed to pendimethalin treatment (Promkaew *et al.,* 2010). Symptoms of short-stature plantlets, characterized by shortening distance between nodes, also emerged in cucumber plantlets treated with pendimethalin (Kennedy *et al.,* 1991). Short-stature plantlets in this study experienced growth retardation as characterized by growth inhibition of plantlet height and size. Pendimethalin is an herbicide that can affect plantlet growth by inhibiting physiological processes and causing short-stature plantlets. This process is detrimental because the treated plantlets need to restore their normal growth status to survive. In this study, high percentages of short-stature plantlets occurred, followed by a low percentage of surviving plantlets. Figure 6 compares the appearance of control, short-stature, and normal plantlets.

**Table 1.** The total number and percentages of chromosomal duplications of haploid plantlets and plantlets with a certain ploidy level (1×, 2×, 3×, 4×, or mixoploid).

| Treatment combination | Sample number | Sample of ploidy checked | Number (in %) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1× | 2× | 3× | 4× | *mixoploid* | NM* |
| Control | 40 | 40 | 40 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 100 µM - 2 day | 40 | 40 | 18 (45.0) | 20 (50.0) | 0 (0) | 1 (2.5) | 1 (2.5) | 0 (0) |
| 100 µM - 4 day | 40 | 37 | 18 (48.6) | 11 (29.7) | 0 (0) | 0 (0) | 1 (2.7) | 7 (18.9) |
| 100 µM - 6 day | 40 | 34 | 14 (41.2) | 13 (38.2) | 0 (0) | 2 (5.9) | 0 (0) | 5 (14.7) |
| 300 µM - 2 day | 40 | 39 | 9 (23.1) | 30 (76.9) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 300 µM - 4 day | 40 | 32 | 17 (53.1) | 14 (43.8) | 1 (3.1) | 0 (0) | 0 (0) | 0 (0) |
| 300 µM - 6 day | 40 | 27 | 9 (33.3) | 16 (59.3) | 0 (0) | 0 (0) | 0 (0) | 2 (7.4) |
| 1000 µM - 2 day | 40 | 35 | 7 (20.0) | 13 (37.1) | 0 (0) | 7 (20.0) | 0 (0) | 8 (22.9) |
| 1000 µM - 4 day | 40 | 25 | 3 (12.0) | 18 (72.0) | 0 (0) | 0 (0) | 0 (0) | 4 (16.0) |
| 1000 µM - 6 day | 40 | 18 | 3 (16.7) | 13 (72.2) | 0 (0) | 0 (0) | 0 (0) | 2 (11.1) |
| Total | 400 | 327 | 138(42.2) | 148(45.3) | 1 (0.3) | 10 (3.1) | 2 (0.6) | 28 (8.6) |

* NM = Not observed = sample was not good and could not be read by flow cytometry.

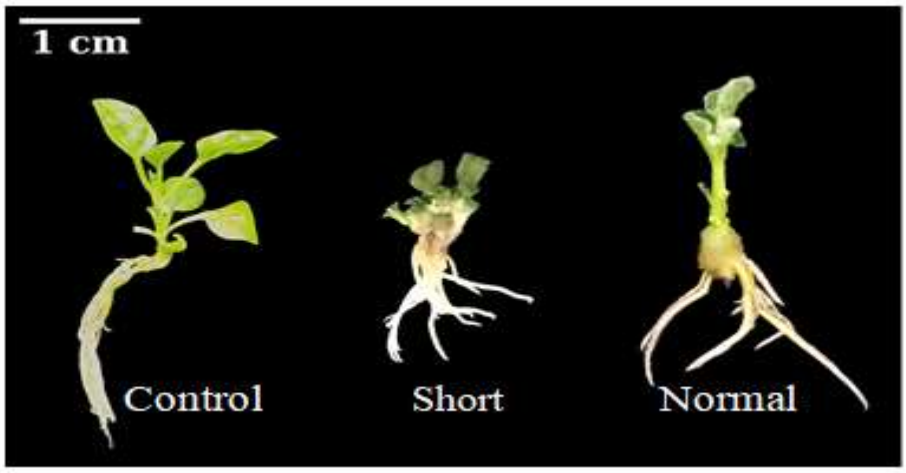


**Figure 6.** Types of eggplant plantlets formed from the treatment of chromosome duplication.

In vitro pendimethalin treatment with a concentration of 30 µM for six days effectively duplicated the chromosomes in onion (Ren *et al.*, 2018). Jiang *et al.* (2020) reported the highest in vitro chromosomal duplication in *Plumbago auriculata* Lam. by using pendimethalin with a concentration of 800 µM and a period of eight days of incubation. In this experiment, a pendimethalin concentration of 300 µM and incubation duration of two days gave the highest number and percentage of chromosomal duplications altering ploidy level status from haploid to doubled haploid in eggplant plantlets. Therefore, it is indicative that pendimethalin treatment will give different results depending on the species. According to Promkaew *et al.* (2010), pendimethalin caused plant genotoxicity. Genotoxicity is the genome alteration due to disturbances in the division and distribution of chromosomes during mitosis.

Pendimethalin can be an alternative anti-mitotic agent that is more effective and inexpensive than colchicine. Başay *et al.* (2011) reported that soaking plantlets with 0.5% colchicine for two hours proved the best method. However, the results of this colchicine method have shown the inability to significantly increase the number of DH plants in eggplant anther cultures. Corral-Martínez and Seguí-Simarro (2012) stated that the application of lanolin paste containing 0.5% colchicine to the axillary shoots of haploid eggplants from anther culture resulted in diploidization rates of 25%. In this experiment, a concentration of 300 µM pendimethalin treatment, with a two-day incubation duration, resulted in 75.0% diploidization rates of eggplant haploid plantlets. Thus, pendimethalin is superior to colchicine as a diploidization agent for haploid eggplants from anther culture. In addition, colchicine is more

expensive than pendimethalin and it may be unaffordable for some laboratories. Colchicine, also known as carcinogenic, usually requires a higher concentration when used as an anti-mitotic agent (0.5% – 1%); thus, it needs special handling by skilled personnel.

## CONCLUSIONS

In vitro chromosomal duplication treatment using pendimethalin at different concentrations and duration of incubation time affected the plantlet survival and growth of eggplant haploid plantlets. The increase in pendimethalin concentration and incubation duration inhibited the physiology and plantlet growth and influenced alteration in the ploidy level of eggplant haploid plantlets. The pendimethalin treatment at a concentration of 300 µM and incubation duration of two days gave the highest level of plantlet diploidization at 75.0%.

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