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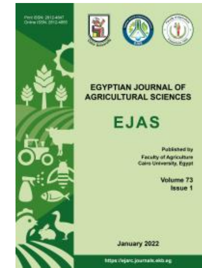

A Comprehensive Protocol for *In Vitro* Propagation and Acclimatization of *Jacaranda mimosifolia* Trees under the Egyptian Conditions

By

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ABSTRACT

This study was carried out during the autumn season of 2021-2022 to establish a detailed protocol for the micropropagation of *Jacaranda mimosifolia* trees. The best results in terms of eliminating contamination (0.0%) were obtained by soaking seeds in Clorox (NaOCl) solution for 9 or 12 min. The highest seeds survival rate (100%) was obtained when sterile seeds were planted in ¾ strength MS medium + 30 g/l sucrose + 6% agar, and pH 5.7. The results showed that ½ strength MS was a suitable medium for the next multiplication stage, because it later gave the highest number of shoots, and chlorophyll A content. On the other hand, the ¼ strength MS medium was suitable for the rooting stage because it resulted in the best root length and shoot length. In terms of shoot length and fresh weight were obtained with 2.0 mg/l 6-BAP + 0.0 mg/l NAA, while 4.0 mg/l 6-BAP + 0.0 mg/l NAA gave the highest number of shoots, and 1.0 mg/l 6-BAP + 0.0 mg/l NAA resulted in the best flavonoids content. The highest root number and dry weight were obtained with 2.0 g/l AC + 0.5 mg/l NAA, while the longest shoots were obtained with 0.0 g/l AC + 0.1 mg/l NAA. In the acclimatization stage, the best plant height was obtained with Peat moss + Perlite at a ratio of 2:1 (v/v). The survival percentage (100%) was equal in both of the tested acclimatization media, viz., Peat moss + perlite at 2:1 (v/v) and Peat moss + perlite + vermiculite at 2:1:1 (v/v/v). The economic feasibility and production costs were calculated after determining the appropriate media for the different micropropagation stages of *J. mimosifolia*.

Keywords: *Jacaranda mimosifolia, micropropagation, in vitro propagation, acclimatization*

1. INTRODUCTION

Jacaranda mimosifolia D. Don (Bignoniaceae) is a thick crown middle-size tree with attractive violet flowers (Encarnacion, 1983). Its native origin is South America. This tree belongs to the family Bignoniaceae, and is most famous in Paraguay, Bolivia, and Argentina. The tropical and temperate climates are suitable to spread and cultivate *J. mimosifolia* (Lorenzi *et al.*, 2003). The value of *J. mimosifolia* comes from being extensively used in decorating urban green spaces and achieving a beautiful design when planted together with *Platanus x acerifolia* (Abutaleb *et al.*, 2021). Tissue culture is a reliable vegetative method for large-scale quick plant propagation under sterile media conditions (Jain and Häggman, 2007). Limiting factors for *J. mimosifolia* propagation include the low germination percentage of seeds (9-35%) which is affected by seeds age (Póvoa, 2018). Also, the woody stem cuttings need to be treated with auxin (IBA) solution for rooting,

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which takes a considerable long time to produce a large tree (Miyajima *et al*., 2004). In contrast, micropropagation of *J. mimosifolia* using *in vitro* culture shortens the period needed to reach out-planting (Maruyama, 2003). The tissue culture micropropagation technique is divided into four stages, the first is the establishment stage, which includes two steps. The first step is sterilization, which reduces contamination and increases seed survival and germination rates (Eneida *et al*., 2018). Clorox (5% NaOCl) and ethyl alcohol (70%) were successfully used for sterilization of *J. mimosifolia* seeds (Maruyama, 2003). The best concentration of Clorox (NaOCl) or $HgCl_2$ should not exceed 3 or 5% (Kim *et al*., 2017). Using the autoclave is the normal process for sterilization of jars (Tisserat *et al*., 1992). The second step is to determine the most suitable culture medium for the next steps of *in vitro* culture. Using Murashige and Skoog (MS) medium was suitable for germination of *Jacaranda* seeds (Eneida *et al*., 2018). In general, using sucrose (3%) as a carbon source is a better choice for *in vitro* medium supplementation (Frey *et al*., 1992). The second stage of micropropagation is multiplication. In this stage, cytokinins and/or auxins are added in a certain balance to help multiplication of explants after several subcultures. It was found from earlier research that using 6-BAP and NAA was successful with *J. mimosifolia* (Maruyama, 2003). The growth of explants on several medium strengths has been examined; the most famous example of all is MS medium at ¼, ½, ¾ and full strength (Abobakr and Ahmed, 2012). Planting of *Jacaranda* stem node explants on ¼ strength MS + 20 g/l sucrose multiplication medium supplemented with 6-BAP and auxin increased shoot number (Eneida *et al*., 2018). The third stage of micropropagation, is rooting, where the culture medium is usually deprived of any added growth regulators, except auxins which are added to encourage and initiate rooting. Also, adding activated charcoal (AC) to the rooting media is useful (Thomas, 2008). Adding auxins (IBA or IBA+NAA) in rooting media of *J. mimosifolia* was suitable for root induction after 1-2 weeks (Maruyama, 2003). Acclimatization stage is the fourth and final stage of *in vitro* micropropagation. In this stage, the rooted explant shoots are removed gradually from the totally controlled *in vitro* culture conditions to the *ex vitro* conditions. The most famous culture media used in acclimatization are peat moss, vermiculite and perlite (Maruyama, 2003 and Abou Dahab *et al*., 2005). The main goal of this research was to investigate the possibility of using tissue culture micropropagation as an alternative method for the mass production of such important tree (*Jacaranda mimosifolia*) that is used extensively for landscape purposes in middle eastern countries like Egypt and Iraq.

2. MATERIAL AND METHODS

2.1. Location and time span

The micropropagation experiment of *J. mimosifolia* was conducted in the tissue culture laboratory, the Genetic Engineering and Biotechnology Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt in autumn, 2021 (12/10/2021).

2.2. Plant material

Fresh seeds of *J. mimosifolia* were taken from a donor tree located in the garden of the Horticulture Department, Faculty of Agriculture, Ain Shams University. These seeds were collected on 23/5/2021 and stored in the refrigerator until planting in autumn of the same year.

2.3. Stages of micropropagation

2.3.1. Establishment stage

The establishment stage was conducted in two steps:

2.3.1.1. Sterilization experiment

The fresh seeds were soaked in two different types of sterilization solutions: common bleach (Clorox, containing 5.25% NaOCl) at a concentration of 10%, and $HgCl_2$ at 0.1 g/l, for three soaking periods (6, 8 and 12 min). The sterile seeds were rinsed three times with sterile distilled water, dried in a laminar hood, then planted directly in flasks containing ¾ strength MS medium + 30 g/l sucrose + 6 g/l agar.

Measured parameters: Contamination and survival percentages.

2.3.1.2. Testing MS medium strength

An experiment was conducted to choose the suitable MS medium strength for growing the stem-node explants. Forty-five days after seeds cultivation, stem node explants were excised and cultivated in MS media of four different strengths (¼, ½, ¾, and full MS strength) + 30 g/l sucrose + 8% agar. The stem node explants had an average size of 1.5-2 cm, and were planted in a 250 ml culture jars containing 60 ml

of the medium (three stem nodes per flask), at a depth of 1 cm, and with ten replicates.

Measured parameters: Shoot length (cm), shoot number, root length (cm), and chlorophyll A concentration (mg/g).

2.3.2. Multiplication stage

Results of the previous stage showed that ½ strength MS medium + 30 g/l sucrose + 8 % agar was a suitable medium for proliferation of *J. mimosifolia* stem node explants. In the multiplication stage, this medium was supplemented with five different concentrations of 6-BAP (0.0, 0.5, 1.0, 2.0, 4.0 mg/l) with or without NAA at 0.1 mg/l. The explants were tried for 60 days.

Measured parameters: Shoot length (cm), shoot number, fresh weight (g), and flavonoids concentration (mg/g).

2.3.3. Rooting stage

The intact multiplied stem node explants of *J. mimosifolia* were planted (without removing the shoot tips) on a ¼ strength MS rooting medium + 15 g/l sucrose + activated charcoal (AC) at 0.0, 0.5, and 2.0 g/l, and NAA at 0.0, 0.1 and 0.5 mg/l. Data were scored after-six weeks from the beginning of the rooting stage.

Measured parameters: Shoot length (cm) dry weight (g) and root number.

2.3.4. Acclimatization stage

The roots of the rooted plants were washed with tap water, then dipped in a fungicide solution (Rhizolex, 0.5 g/l) for 30 minutes. Then, the plants were planted in two plastic pots filled with sterile Peat moss + Perlite (2:1, v/v), and Peat moss + Perlite + Vermiculite (2:1:1, v/v/v). Each treatment contained 3 replicates. The plants were then irrigated with filtered water and transferred (on 12/7/2022) to a greenhouse, where acclimatization continued for 60 days under high humidity (85%) and an average temperature of 34° C.

Measured parameters: Plant height (cm) and survival percentage.

2.4. Chemical analysis

2.4.1. Determination of chlorophyll A concentration

Chlorophyll A was extracted using the method described by Metzener *et al*. (1965), by grinding 0.5 g of fresh *J. mimosifolia* leaves (taken from the establishment stage) with acetone (85%), then pouring into a test tube and adding 7 ml of acetone to dissolve the chlorophyll A pigment. This mixture was put in a hot water bath at a temperature of about 65° C for 15 min, then it was filtered by centrifuging. The resulting supernatant was pipetted into a clean tube, and light absorption was measured using a colorimeter at a wavelength of 664 nm. The chlorophyll A concentration in the solution was determined using the following equation:

Chlorophyll A =10.3 A664 - 0.918 A645 g/ml

The concentration of chlorophyll A in plant fresh matter was calculated as follows:

$$\text{Chlorophyll concentration (mg/g)} = \frac{Fraction \times dilution}{1000}$$

2.4.2. Determination of flavonoids concentration

The aluminum chloride colorimetric technique was used to extract and measure total flavonoids concentration in *J. mimosifolia* plants resulting from the multiplication stage (Zhishen *et al.,* 1999). This extract was mixed with 150 ml of 5% $NaNO_3$ then let stand for 6 minutes, followed by adding 150 ml of 10% $AlCl_3$ solution and letting it stand for 6 minutes. Then, 200 ml of a NaOH solution + 5 ml methanol were added and mixed thoroughly. The absorbance was measured spectrophotometrically against a blank at 510 nm after 15 minutes of incubation. The total flavonoid content was calculated in milligrams of quercetin equivalents (QE) per gram of extract (mg QE/g). The total was calculated using the quercetin standard curve, according to the following equation:

Concentration (mg/g) = ((R-B)* dilution factor*factor)/1000.

2.5. Experimental design and statistical analysis

The experimental design was a complete randomized design and the data recorded were statistically analyzed using IBM SPSS Version 20 (SPSS Inc., Chicago, USA) and the means were compared by using the Tukey HSD test at a 5% significance level.

3. RESULTS AND DISCUSSION

3.1. Establishment stage

3.1.1. Effect of sterilization treatments

The results of the sterilization experiment (Table 1) showed that the highest contamination percentage (30%) was obtained with soaking the

seeds in 0.1 g/l $HgCl_2$ for 6 minutes. No contamination (0.0%) was recorded when Clorox' (5.25% NaOCl) was used at 10%, for soaking periods of 9 or 12 min.

The results presented in Table (1) also show that the highest survival percentage (100 %) was achieved with 10% NaOCl for 9 or 12-min soaking periods, whereas the lowest survival percentage (70%) was obtained with 0.1g/l $HgCl_2$ with a 6-min soaking period.

Table (1): Effect of sterilization treatments on *J. mimosifolia* seeds during *in vitro* establishment stage.

Seeds contamination percentage (%)				Survival percentage (%)		
Sterilization treatments	**Sterilization periods**			**Sterilization periods**		
	6 min	9 min	12 min	6 min	9 min	12 min
10% NaOCl	1^{d}	0^{e}	0^{e}	99^{b}	$\mathbf{100^{a}}$	$\mathbf{100^{a}}$
0.1g/l $HgCl_2$	$\mathbf{30^{a}}$	6^{b}	2^{c}	70^{f}	94^{d}	98^{c}

3.1.2. Effect of MS medium strength

3.1.2.1. Shoot length (cm)

The data presented in Table (2) show that the longest shoots (3.167 cm) were obtained with ¼ strength MS medium, followed by shoots with lengths of 2.933 and 2.833 cm which were obtained with full strength MS or ½ strength MS, respectively. The shortest shoots (2.367 cm) were obtained with ¾ strength MS medium.

3.1.2.2. Shoot number

It can be seen from the data in Table (2) that the highest number of shoots (6.0/explant) was obtained with ½ strength MS, whereas all the remaining treatments gave the same number of shoots (3.0/explant).

3.1.2.3. Root length (cm)

The recorded results (Table 2 and Fig.1) show that only the ¼ strength MS medium induced the formation of roots with a root length of 4.0 cm. Meanwhile, all the remaining treatments resulted in rootless explants.

3.1.2.4. Chlorophyll A concentration (mg/g)

As shown by the data in Table (2), the highest content of chlorophyll A (0.229 mg/g) was obtained with ½ strength MS, followed by 0.214 mg/g which was obtained with ¾ strength MS. On the other hand, the least chlorophyll A concentration (0.195 mg/g) was obtained with full strength MS, and that was insignificantly different than the concentration recorded with ¼ strength MS medium.

3.2. In vitro multiplication stage

3.2.1. Shoot length (cm)

As shown by the data in Table (3), the longest shoots (4.40 cm) were obtained with 2.0 mg/l 6-BAP, followed by shoots that were obtained with 0.1 mg/l NAA (with a length of 3.37 cm). The shortest shoots (0.87 cm) were obtained with 4.0 mg/l 6-BAP.

3.2.2. Shoot number

The results presented in Table (3) also show that the highest number of shoots (16.0) was obtained with T_6, followed by 12.0 that was obtained with T_3. On the other hand, the lowest number of shoots was obtained in the control and with 0.1 mg/l NAA. These two treatments gave a significantly lower number of branches (1.0) than any of the other treatments.

3.2.3. Fresh weight (g)

The recorded results (Table 3 and Fig. 2) show that the heaviest fresh weight (0.45 g) was obtained with 0.1 mg/l NAA or 2.0 mg/l 6-BAP, followed by the control or 1.0 mg/l 6-BAP, with a fresh weight of 0.35 g, which is still heavier than those recorded with any of the remaining treatments. The least fresh weight (0.10 g) was obtained with 4.0 mg/l 6-BAP + 0.1 mg/l NAA.

3.2.4. Flavonoids concentration (mg/g)

Regarding the effect of the treatments on the flavonoid's concentration, the data in Table (3) show that the highest concentration (0.686 mg/g) was obtained with 1.0 mg/l 6-BAP, followed by 0.500 mg/g which was obtained with 2.0 mg/l 6-BAP. The lowest value (0.003 mg/g) was recorded with 0.1 mg/l NAA which was more or less equal to most of the remaining treatments.

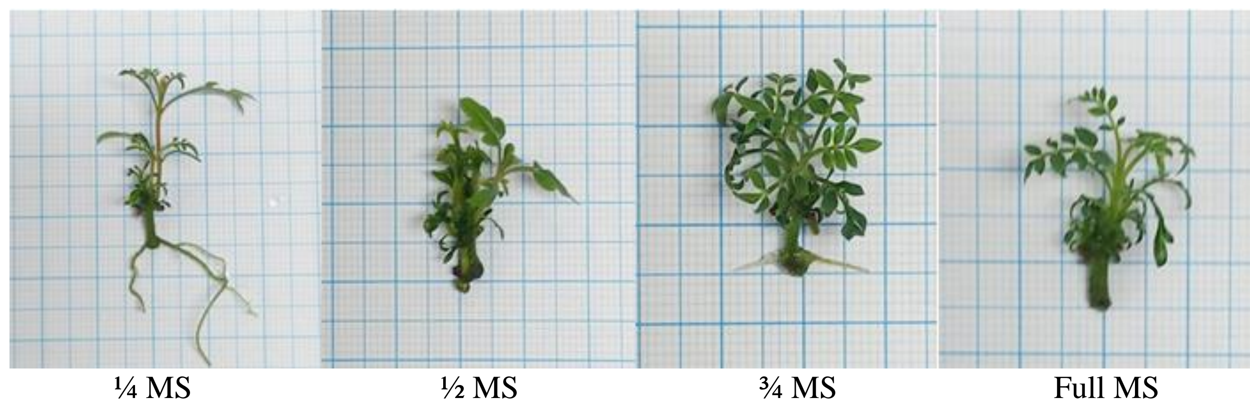
3.3. Rooting Stage

3.3.1. Shoot length (cm)

From the data shown in Table (4) it is clear that the longest shoots (6.66 cm) were obtained with

Table (2): Effect of MS medium strength on shoot length, shoot number, root length and chlorophyll A concentration during *in vitro* establishment stage of *J. mimosifolia*

Treatments	MS strength (g/l)	Shoot length (cm)	Shoot number	Root length (cm)	Chlorophyll A mg/g
T_1	¼ (1.1 g/l)	**3.167^{a}**	3.0^{b}	**4.00^{a}**	0.201^{c}
T_2	½ (2.2 g/l)	2.833^{b}	**6.0^{a}**	0.00^{b}	**0.229^{a}**
T_3	¾ (3.3 g/l)	2.367^{c}	3.0^{b}	0.00^{b}	0.214^{b}
T_4	Full-(4.4 g/l)	2.933^{b}	3.0^{b}	0.00^{b}	0.195^{c}



¼ MS ½ MS ¾ MS Full MS

Fig.1. Effect of MS medium strength on *J. mimosifolia* during the establishment stage.

Table (3): Effect of different 6-BAP and NAA concentrations on shoot length (cm), shoot number, fresh weight (g) and flavonoids concentration (mg/g) during *in vitro* multiplication stage of *J. mimosifolia*.

Treatments 6-BAP and NAA Concentrations (mg/l)	Shoot Length (cm)	Shoot number	Fresh weight (g)	Flavonoids (mg/g)
T_1 (0.0 6-BAP + 0.0 NAA) (Control)	2.83^{d}	1.00^{g}	0.35^{b}	0.014^{d}
T_2 (0.0 6-BAP + 0.1 NAA)	3.37^{b}	1.00^{g}	**0.45^{a}**	0.074^{c}
T_3 (0.5 6-BAP + 0.0 NAA)	1.00^{g}	12.00^{b}	0.22^{c}	0.012^{d}
T_4 (1.0 6-BAP + 0.0 NAA)	3.00^{c}	4.00^{e}	0.35^{b}	**0.686^{a}**
T_5 (2.0 6-BAP + 0.0 NAA)	**4.40^{a}**	5.00^{d}	**0.45^{a}**	0.500^{b}
T_6 (4.0 6-BAP + 0.0 NAA)	0.87^{i}	**16.00^{a}**	0.22^{c}	0.009^{d}
T_7 (0.5 6-BAP + 0.1 NAA)	2.07^{e}	4.00^{d}	0.11^{d}	0.003^{d}
T_8 (1.0 6-BAP + 0.1 NAA)	0.93^{h}	2.00^{f}	0.11^{d}	0.005^{d}
T_9 (2.0 6-BAP + 0.1 NAA)	1.00^{g}	4.00^{e}	0.11^{d}	0.006^{d}
T_{10} (4.0 6-BAP + 0.1 NAA)	1.20^{f}	8.00^{c}	0.10^{e}	0.011^{d}

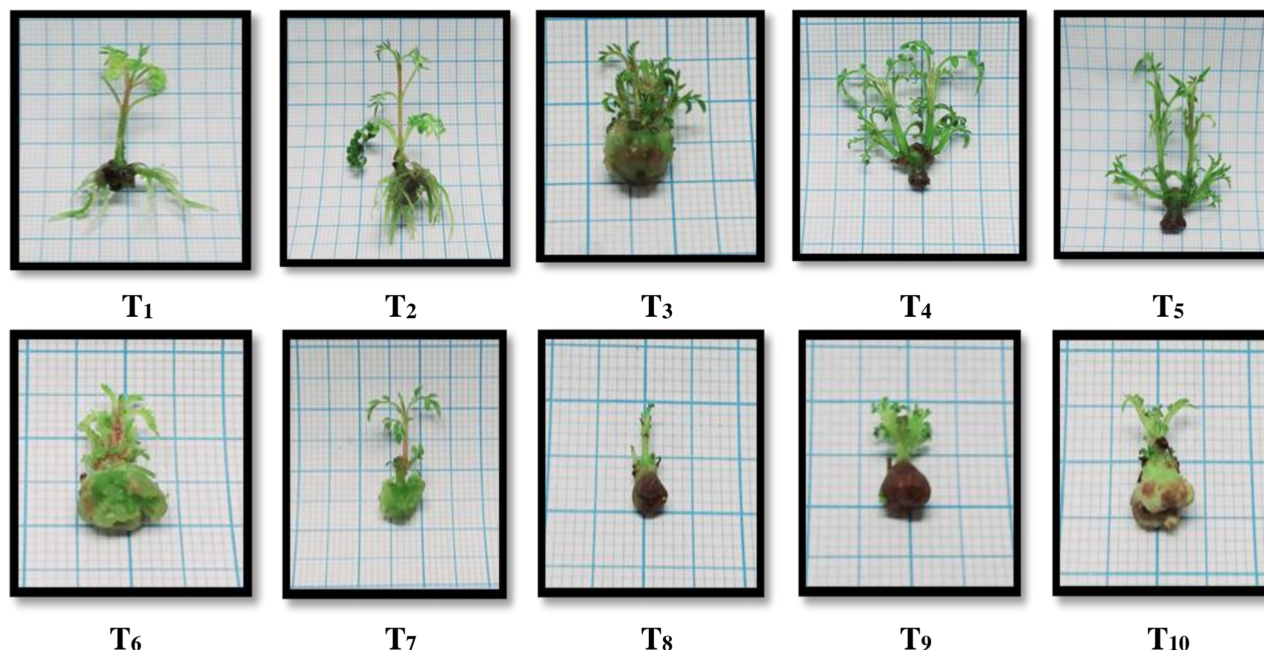


Fig. 2. Effect of different 6-BAP and NAA concentrations during the multiplication stage of *in vitro* micropropagation of *J. mimosifolia* (letters T1 to T10 are described in the text and Table 3).

0.0 g/l AC + 0.1 mg/l NAA, followed by 2.0 g/l AC + 0.0 mg/l NAA, T_7 0.5 g/l AC + 0.5 mg/l NAA and 2.0 g/l AC + 0.1 mg/l NAA (which all gave a shoot length of 4.00 cm). On the other hand, the shortest shoots (2.00 cm) were obtained with 0.0 g/l AC + 0.5 mg/l NAA.

3.3.2. Dry weight (g)

The data presented in Table (4) show that the heaviest dry weight (0.90 g) was obtained with 2.0 g/l AC + 0.5 mg/l NAA, followed by a dry weight of 0.80 g, that was obtained with 0.0 g/l AC + 0.1 mg/l NAA. The lightest dry weight (0.1 g) was obtained with 0.5 g/l AC + 0.0 mg/l and 2.0 g/l AC + 0.0 mg/l NAA.

3.3.3. Root number

The recorded results (Table 4 and Fig. 3) show that the highest root number (21.0) was obtained with 2.0 g/l AC + 0.5 mg/l NAA, followed by 12.0 which was obtained with 0.0 g/l AC + 0.1 mg/l NAA. The lowest number of roots (0.0) was obtained with 0.0 g/l AC + 0.0 mg/l NAA, 0.5 NAA.

Table (4): Effect of different concentrations of active charcoal (AC) and NAA on shoot length (cm), dry weight (g) and root number during *in vitro* rooting stage of *J. mimosifolia*.

Treatments (AC and NAA Concentrations)	Shoot length (cm)	Dry weight (g)	Root number
T_1 0.0 g/l AC + 0.0 mg/l NAA (Control)	3.00^{c}	0.03^{d}	0.00^{f}
T_2 0.5 g/l AC + 0.0 mg/l NAA	3.00^{c}	0.01^{f}	0.00^{f}
T_3 2.0 g/l AC + 0.0 mg/l NAA	4.00^{b}	0.01^{f}	0.00^{f}
T_4 0.0 g/l AC + 0.1 mg/l NAA	**6.66^{a}**	0.80^{b}	12.00^{b}
T_5 0.0 g/l AC + 0.5 mg/l NAA	2.00^{d}	0.02^{e}	7.00^{d}
T_6 0.5 g/l AC + 0.1 mg/l NAA	3.00^{c}	0.02^{e}	7.00^{d}
T_7 0.5 g/l AC + 0.5 mg/l NAA	4.00^{b}	0.20^{c}	8.00^{c}
T_8 2.0 g/l AC + 0.1 mg/l NAA	4.00^{b}	0.20^{c}	4.00^{e}
T_9 2.0 g/l AC + 0.5 mg/l NAA	2.66^{cd}	**0.90^{a}**	**21.00^{a}**

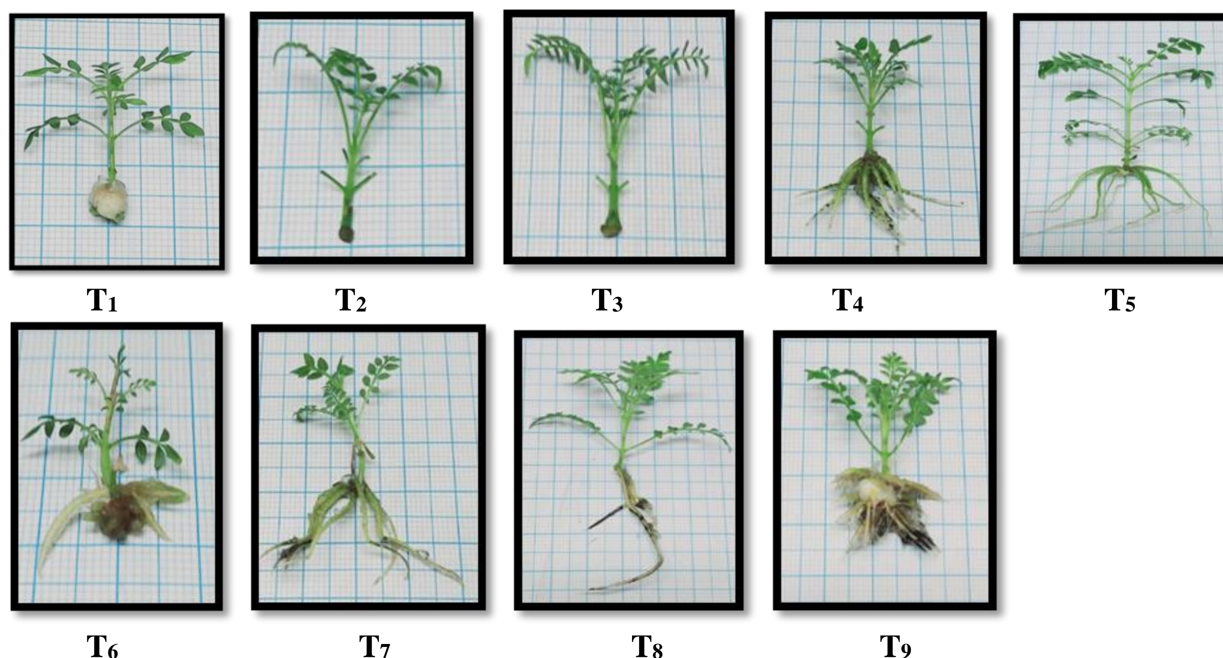


Fig. 3. Rooting stage of *in vitro* micropropagation of *J. mimosifolia* (letters T_1 to T_9 are described in the text and Table 4).

3.4. Acclimatization stage

3.4.1. Plant height (cm)

The data in Table (5) show that the tallest plants (16.87 cm) were obtained with T_1, followed by a height of 15.0 cm, which was obtained with T_2.

3.4.2. Survival percentage

As shown by the data in Table (5), the maximum survival percentage (100%) was recorded with both 2 Peat + 1 Perlite and 2 Peat + 1 Vermiculite + 1 Perlite.

3.5. General discussion

In the *in vitro* experiment of *J. mimosifolia* seeds, soaking the seeds in Clorox at 10% concentration for periods of **6,** 8 or 12 min totally eliminated any contamination (0.0% contamination) and increased survival to its maximum level (100%). Similar results were obtained earlier by Maruyama (2003) on *J. mimosifolia*, Grevenstuk *et al.* (2010) on *Drosera intermedia*, Surendranath *et al.* (2016) on tuberose, and Eneida *et al.* (2018) on *Jacaranda ulei*. However, other dissimilar results were obtained by Safarnejad and Saeedi (2015) who reported that soaking seeds of *Acer monospessulanum* in 0.1% $HgCl_2$ for three minutes, ethanol 70% for three minutes, and NaOCl for twenty minutes gave the best result for seeds sterilization.

Using ¼ strength MS medium in the *in vitro* establishment stage improved both shoot length and root length of *J. mimosifolia*. Similar finding were reported by Tarinejad and Amiri (2019) on *Vitis vinifera* L. cv. Shahroudi, but Taha *et al*. (2009), on Garden Balsam (*Impatiens balsamina*), found that hormone-free full strength MS medium gave the best result in terms of shoot length.

Using ½ strength MS medium in the *in vitro* establishment stage increased shoot number and chlorophyll A content inside growing stem nodes explants of *J. mimosifolia.* Similar results were obtained by Abou Dahab *et al*. (2005) on *Ruscus hypoglossum*, and Ezequiel *et al*. (2012) on Pink Lapacho.

In the *in vitro* multiplication stage of established stem node explants of *J. mimosifolia*, using ½ strength MS medium, supplemented with only 2.0 mg/l 6-BAP (without NAA), increased both shoot length and fresh weight. This is in agreement with results reported by Tariq *et al.* (2006) on *Bougainvillea spectabilis*, Niu and Cao (2009) on *Spiranthes sinensis*, and Abou Rayya *et al.* (2010) on bitter almond. In contrast, Vaidya *et al.* (2019) on *Mentha piperita* L. found that using full strength MS medium with 4.0 mg/l 6-BAP gave the highest shoot numbers.

In this study, when 6-BAP concentration was doubled to 4.0 mg/l (without NAA) shoot numbers were increased. A similar result was obtained by Abou Rayya *et al.* (2010) on bitter almond.

Table (5): Effect of different acclimatization media on plant height and survival percentage of *J. mimosifolia* plants after 60 days.

	Acclimatization results	
Treatments (Acclimatization media)	Plant height (cm)	Survival percentage %
T_1 (2 Peat + 1 perlite)	**16.87[a]**	100
T_2 (2 Peat + 1 vermiculite + 1 Perlite)	15.0[b]	100

However, a different conclusion was reached by Milena (2012) who found out that 1 mg/l 6-BAP gave the highest shoot numbers of *Jacaranda decurrens*, and Cardoso & da Silva (2013) on *Zehria Montana,* who found out that ¼ strength MS medium + 1 mg/l 6-BAP obtained highest buds per/ explant.

When 6-BAP concentration was lowered to 0.1 mg/l (still without any NAA), the flavonoids content was raised inside multiplied shoots of *J. mimosifolia*. Similar results have been reported by Vaidya *et al.* (2019) on *Mentha piperita* L., who found that total flavonoid content increased by *in vitro* culture of *Mentha piperita*, compared to cultivation under greenhouse conditions.

In the *in vitro* rooting stage, using ¼ strength MS medium supplemented with 15 g/l sucrose + 2.0 g/l AC + 0.5 mg/l NAA increased root number and dry weight. Similar results were obtained by Demiralay *et al.* (1998) on *Ficus carica*, and by Zakaria *et al.* (2020) on *Scutellaria Alpina* and *S. altissima.* When AC was totally eliminated from the *in vitro* growing medium only shoot length was positively affected. This result is in agreement with the findings of Ruffoni *et al.* (2013) on *Hydrangea macrophylla*.

In the acclimatization stage of *J. mimosifolia* rooted plantlets, both culture media gave an excellent survival percentage, except that the peat moss: perlite (2:1 v/v) medium was preferable as it increased plant height. That is why it is recommended for acclimatization of *J. mimosifolia*. A similar result was obtained on *Phoenix dactylifera* L. var. Maktoom by Khierallah and Bader (2007).

3.6. Interpretation of results

Contamination with bacteria during *in vitro* propagation delays the development and growth of micropropagated plant organs, either because of the toxic effect or from using up the nutrients in the culture media. To avoid these negative effects sterilization is carried out to obtain a better survival rate and to achieve decent growth (Singh *et al.,* 2019).

When common bleach 'Clorox' is used to sterilize *in vitro* culture HOCl is formed as a result of dilution in water and OCl-ions are released which have a direct detrimental effect on the cells of bacteria causing contamination (Webster and Mitchell, 2003). Also, mercuric chloride ($HgCl_2$) is used in *in vitro* sterilization where the Hg-ion is released and is highly toxic against bacteria, as it is absorbed quickly into bacteria cell causing death (Hashim *et al.*., 2021).

Since plants are living organisms, they differ in their response to sterilizing chemicals (NaOCl and/or $HgCl_2$) according to their plant type and their natural sensitivity to such chemicals (Akin-Idowu *et al.*, 2009 and Hashim *et al.*, 2021). As it turned out to be, seeds of *J. mimosifolia* responded more positively to NaOCl rather than $HgCl_2$ when used during sterilization.

Murashige and Skoog medium was and still is the most widely used medium for *in vitro* culture trials (Murashige and Skoog, 1962). It can be used in either ¼, ½, ¾ or full strength according to explants type and/or *in vitro* stage of growth. From preliminary trials with *J. mimosifolia* it was found that ½ strength MS was more suitable for the *in vitro* multiplication of established stem node explants. However, in the *in vitro* rooting stage ¼ strength was more appropriate. This makes sense, as during the multiplication stage more nutrients are required to boost growth and number of stem shoots, whereas during the rooting stage less nutrients are required by the individual multiplied shoots which depend primarily on auxins for their rooting process.

Starting from the multiplication stage, cytokinins and auxins are usually added in a certain balance to encourage growth and proliferation of the established explants. Cytokinins (like BA and 6-BAP) are well known to cause cell division, encourage lateral branching and remove apical dominance (Singh *et al.*, 2021).

Synthetic growth regulators (like BAP and NAA) are supplemented in the *in vitro* culture media during the multiplication stage to make up

for the lack of natural hormones (Ziatin and IAA) in the established explants, thus promote greatly their multiplication ability. This was what exactly happened when the established stem nodes of *J. mimosifolia* were supplemented with 2.0 mg/l and 4.0 mg/l 6-BAP, i.e., the added growth regulators increased shoot length and fresh weight (with 2.0 mg/l) and shoot number (4.0 mg/l). Notably, it must be stressed that the decrease of auxin concentration or total lack of it in the *in vitro* multiplication media could lead in some cases to the same effect as auxin movement in the opposite direction and counteraction of cytokinins. This occurred in this study with *J. mimosifolia* when the multiplication medium was deprived from NAA, which caused more positive results.

During the *in vitro* rooting stage, auxins are usually applied to help initiation of adventitious roots on the multiplied shoots. Also, sucrose is added to the rooting medium as a source of carbon to help plant cell growth. Sucrose added in *in vitro* culture acts as an energy source for supporting phototrophic metabolism, hence enhancing ideal growth (Coupe *et al.*, 2006 and Muller *et al.,* 2011).

Moreover, activated charcoal (AC) is added to the rooting media as it has a critical three-way role. First, it assists in getting rid of phenolic exudates. Second, it overcomes the dark brown color accumulation in the growing media. Third, it helps to adsorb mineral ions, vitamins,–plant growth regulators (auxins), thus promoting development and growth (Thomas, 2008).

According to the abovementioned explanation, using 2.0 g/l AC + 0.5 mg/l NAA + 15 g/l sucrose on multiplied shoots of *J. mimosifolia* during the *in vitro* rooting stage, caused a dramatic increase in root numbers.

As for the dual *in vitro*/ *ex vitro* acclimatization stage, two culture media were used, viz., peat moss + perlite (2:1 v/v) and peat moss + perlite + vermiculite (2:1:1, v/v/v). The role of peat moss in the acclimatization medium was as a source of decomposed organic matter. Both vermiculite and perlite were used to improve wetness holding and ventilation of the growing medium (Fig. 4).

T2 *In vitro* under laboratory conditions **T1 *In vitro* under laboratory conditions**

***Ex vitro* under greenhouse conditions.**

Fig. 4. Dual *in vitro* and *ex vitro* acclimatization stage of *J. mimosifolia*.

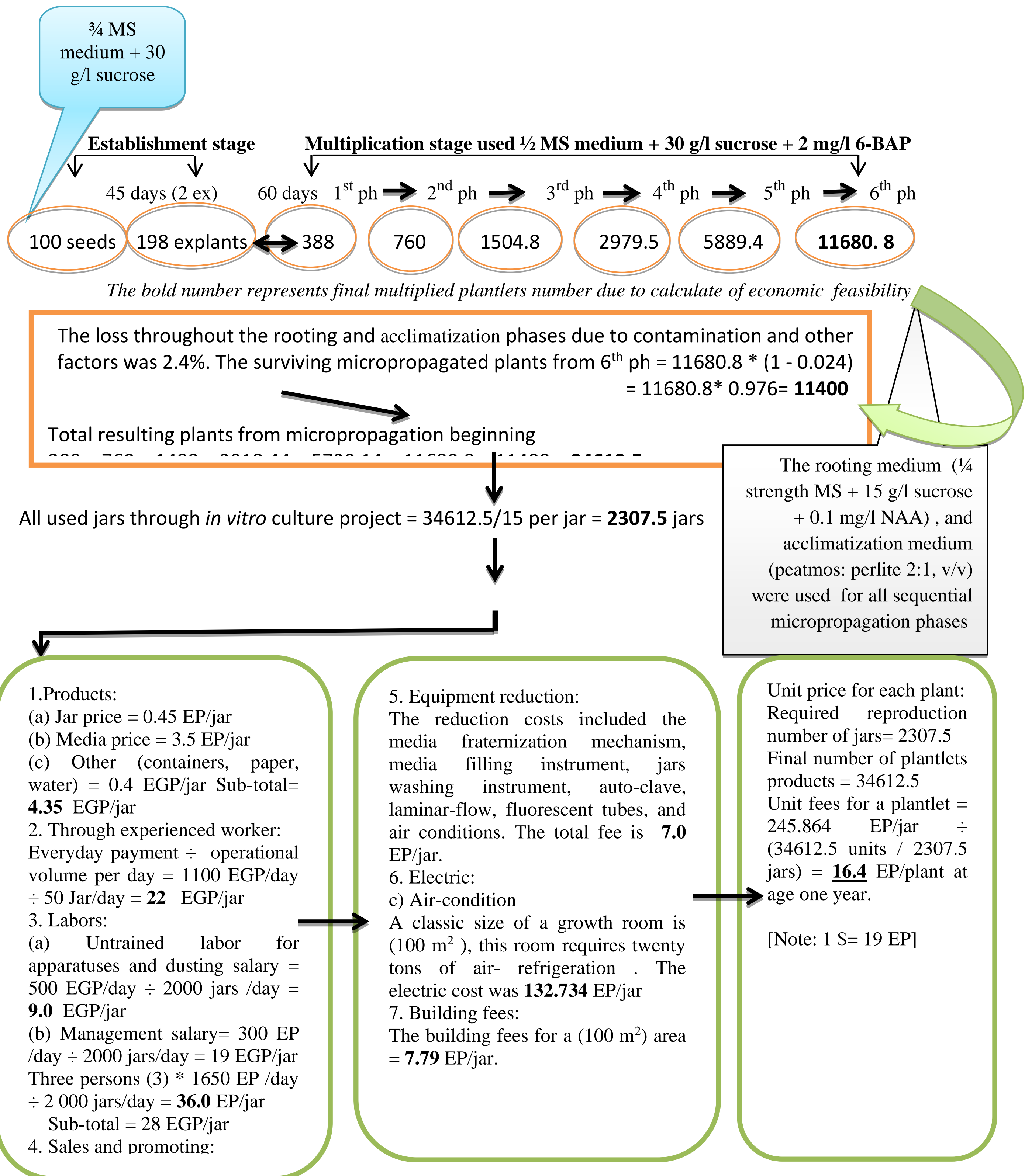


Fig. 5. Economic feasibility study of costs and return of micropropagation of *J. mimosifolia* in Egypt during 2021-2022 (in Egyptian currency).

Peat moss consists of organic materials that have been exposed to some level of decomposition, which gives it a suitable pH (5.5-8), and turns it into a water-logging anoxic environment (Crum, 1992).

Perlite consists of formless volcanic crystals that have a high water-holding level. It is useful for its light-weight, and has a chemical structure of 70-75% SiO_2, 3-4% Na_2O, 12-15% Al_2O_3, 0.5-2% Fe_2O_3, 3-5% K_2O, 0.5-1.5% CaO, 0.2-0.7% MgO and 3-5% inert nonflammable components. Perlite can be replaced by expanded clay, diatomite, shale pumice, vermiculite or slag (Samar and Saxena, 2016).

Vermiculite is an Al-Fe-Mg inorganic silicate characterized by its high nutrient content and water holding capacity. It has high contents of potassium, calcium, and magnesium (Ferry *et al.*, 1998).

The first medium in the acclimatization stage (Peat moss + perlite, 2:1, v/v) was better than the second medium (Peat moss + perlite + vermiculite, 2:1:1, v/v/v) because of its high content of organic matter (2/3 peatmoss) compared to (1/2 peatmoss) in the second medium.

3.7. Conclusions and recommendations

From the results of this study, the following conclusions could be deduced, and can be considered as a basis to creating a rather comprehensive protocol for the micropropogation of *Jacaranda mimosifolia*:

- Common Clorox can be used at a concentration of 10% to sterilize seeds.
- MS medium at ¾ strength + sucrose 30 g/l + agar 6% is suitable for seed germination.
- MS medium at ¼ strength is optimum for rooting stage.
- Using ½ strength MS medium is best choice for multiplication stage.

The economic feasibility was calculated when the multiplied explants reach to ≥10,000 units. One explants proliferation need 2 months for re-culture and the evaluation started after 6 subcultures. The cost of production is estimated by calculating the number of flasks used from beginning of micropropagation and their cost, then dividing by the number of explants produced.

The basic formula and information of the economic feasibility study was adopted from Chen (2016).

- Addition of 6-BAP at 2.0 mg/l is extremely beneficial in the multiplication stage and is highly favored.
- Adding NAA at 0.1 mg/l in the multiplication medium did not benefit all parameters.
- Using NAA at 0.5 mg/l and AC at 2 g/l is highly recommended during the rooting stage, to improve root dry weight and number of roots.
- Using peat moss + perlite (2:1, v/v) as a culture medium during the acclimatization stage results in healthy plants

The abovementioned conclusions can be safely recommended after taking into consideration the costs and returns, i.e., the feasibility study for the micropropagation of *J. mimosifolia* (Fig. 5).

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بروتوكول شامل للإكثار المعملي لأشجار الجكرندا ميموسيفوليا وأقلمتها تحت الظروف المصرية

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ملخص

أجريت الدراسة لإنشاء بروتوكول تفصيلي للاكثار الدقيق لشجرة الجكرندا (*Jacaranda mimosifolia*). أثبتت النتائج أن أفضل النتائج من حيث منع التلوث بنسبة تلوث 0.0 % تحققت عند نقع البذور في محلول الكلوروكس للفترتين 9 و 12 دقيقة. كما أن أعلى نسبة بقاء البذور حية (100%) تحققت عند زراعة تلك البذور المعقمة على بيئة موراشيج وسكوج (MS) بقوة ¾ + 30 جم /لتر سكروز + 6 جم /لتر آجار ودرجة حموضة 5.7. أوضحت النتائج أن البيئة ½ قوة أملاح موراشيج وسكوج MS كانت بيئة مناسبة للتضاعف، لذلك اختيرت هذه القوة لمرحلة التضاعف لأنها أعطت أفضل عدد النبيتات ونسبة محتوى الكلوروفيل أ فى هذه الدراسة. من ناحية أخرى، أظهرت نفس البيئة بقوة ¼ أنها مناسبة في مرحلة الجذور حيث أدت إلى أفضل طول النباتات والجذور. أما بالنسبة لطول النبيتات و الوزن الطازج فكانت عند تركيز 2.0مجم /لتر بنزايل أمينو بيورين بينما عند استعمال البنزايل أمينو بيورين بتركيزات مختلفة (4.0 مجم /لتر) مع إضافة نفثالين حامض الخليك بتركيز (0.0 و 0.1 مجم /لتر أعطت أكبر عدد من النبيتات مع تركيز 4.0مجم /لتر بنزايل أمينو بيورين. بينما اظهرت المعاملة بتركيز 1.0 مجم /لتر بنزايل أمينو بيورين تفوقا في محتوى الفلافونويد. كما أن أكثر عدد من الجذور عند استعمال 2.0 جم /لتر فحم نشط + 0.5 مجم /لتر نفثالين حامض الخليك. بينما أدت المعاملة ب 2جم /لتر فحم نشط +0.1 مجم/لتر نفثالين حامض الخليك أفضل النتائج من حيث عدد الجذور والوزن الجاف، في حين تم الحصول على أطول الجذور في حالة المعاملة الخالية من الفحم النشط + 0.1 مجم/لتر نفثالين حامض الخليك. وفي مرحلة الأقلمة تم الحصول على أعلى ارتفاع للنبات مع استعمال مخلوط من بيئة الزراعة المكونة من (بيتموس+ بيرلايت بنسبة 1:2، حجم/حجم) و تم الحصول على نسبة بقاء 100% في البيئتين (بيتموس+ بيرلايت 1:2، حجم/حجم) و (بيتموس+ بيرلايت+ فيرميكولايت بنسبة 1:1:2، حجم/حجم/حجم). تم حساب الجدوى الاقتصادية وتكاليف الانتاج بعد تحديد استعمال البيئات الزراعية الملائمة لجميع مراحل الإكثار لشجرة الجكرندا ميموسوفوليا.

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