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ESTABLISHMENT OF HIGHLY REPRODUCIBLE PROTOCOL OF HARMAL (*PEGANUM HARMALA* L.) THROUGH *IN VITRO* PROPAGATION

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Peganum harmala L. is a perennial wild herb belonging to the family Zygophyllaceae, it was recently added into the Nitraraiceae family (Zhang and Chi, 2019). *Peganum harmala*, is communally known as harmal, esfand, wild rue or syrian rue (Ghasemi *et al.*, 2014). It exists in large quantities in Europe, North Africa, the Middle East, Central Asia, Northwest India, and Northwest China (Li *et al*., 2017). This plant has 5–13 stems that are branching, and its leaves are plasticated into 3-5 linear lobes that are 1.5-3.0 mm wide and 3-6 cm long. The fruits are globular capsules with three chambers that are 0.9-1.3 cm in diameter and contain 35–45 angular blackish seeds. Flowers appear in groups of one to three at the tips of branches and have whitish–yellow petals (Shahrajabian *et al*., 2021). In Egypt, plant communities are primarily found in the Mediterranean coastal strip, Eastern desert and Sinai. In addition, it's disturbed in empty spaces, ground and

beside roadway (El-Bakatoushi *et al*., 2011).

The major therapeutic effects of harmal include the prevention of candidiasis, anti-inflammatory, anticholinesterase, anti-bacterial, anti-microbial, anti-tumor, angiogenesis, antiparasitic, antioxidant, and cytotoxicity activities, as well as the potential for abortion and pesticide effects (Majid, 2018). The most significant traditional uses of *P. harmala* include treating rheumatism, arthritis, inflammation, diabetes, gastrointestinal, neurological, endocrine, neoplasm and tumors, pain relief, and skin and hair problems (Wang *et al*., 2016). This multifunctional medicinal plant possesses a variety of phytochemical characteristics and a variety of active alkaloids, especially beta-carbolines like harmalol, harmaline as well as harmine (Shahrajabian *et al*., 2021).

Harmal is propagated in nature with seeds .It faces several limitations in germination due to the short span seeds viability. There is no accurate information on the plant's seed germination, maturity, and fruiting in natural environments. Additionally, habitat degradation and over harvesting for use it as a traditional remedy by publics of the wild. The risk of extinction is higher because the plant grows wild rather than being cultivated. (Khawar *et al*., 2005).

In terms of economic input, the preservation and sustainable use of this plant is essential to save this unique medical plant. The technique of *in vitro* culture offers an enormous opportunity for its preservation through multiplication. Micropropagation and *in vitro* culture are biotechnological techniques that are crucial for *ex situ* preservation of the endangered medicinal plants (Goyal *et al*., 2014). The plant crops and endangered species have been previously propagated using *in vitro* strategy (Reed *et al*., 2011). In addition to the independence from seasonality, rapidity, and ability, to generate pathogen-free plants (Debnath *et al*., 2006). Growth regulators, including auxins and cytokinin, are an essential component in controlling the growth and effectiveness of *in vitro* plant culture (Al Khateeb *et al*., 2017). Therefore, the main objectives of this study were establishment of a rapid and reliable protocol in order to produce large number of the *Peganum harmala* L. and identify the optimal conditions for each stage in the micropropagation process.

MATERIAL AND METHODS

Plant material: *Peganum harmala* L. mature seeds were collected from Waddi Feran, Sinai. (Nov 2021).

Seeds sterilization and germination

Surface sterilization of seeds was performed by immersion in 100% commercial Clorox (5%NaOCl) for 5 minutes. Seeds rinsing three times by soaking in sterile distilled water for 3 min and thrown out water. Sterilized seeds were cultured onto MS medium (Murashige and Skoog, 1962) without and/or with different concentration of 6-

benzylaminopurine (BAP, Table 1), 3% sucrose and 0.8% agar, pH was adjusted at 5.8. Cultures were incubated at 25±20 °C with 16/day photoperiod for 2-3 weeks. Data were recorded as the germinated and verified seedlings percentages.

Shoot multiplication

Shoot tips (SHT-EX) and cotyledonary nodes (CN-EX) as explants were carried out in this study. Explants were excised from 15-day-old *in vitro* seedlings that grown on different germination medium. Subsequently, cultured on different multiplication media based on MS supplemented with different concentration of BAP and/or 1-Naphthaleneacetic acid (NAA, Table 2), and incubated for 3-4 weeks under the same growth room conditions to determine the optimal medium for multiplication. The best medium was chosen depended on number of shoots/explants, the shoot length and callus absence. Each treatment was replicated three times with 15 explants/replicate. Data were statistical analyzed by GraphPad Prism9.4.1 (681) program based on analysis of variance (ANOVA).

Root formation

Obtained shoots transferred into different media composed of full or half MS strength medium supplemented with indole-butyric acid (IBA, 0;0.5;1;1.5 mg/l), indole-3-acetic acid (IAA, 0;0.5;1;1.5 mg/l) or NAA (0;0.5;1;1.5 mg/l). Cultures were kept under growth room conditions as described before for 2-4 weeks. Shoots did not form roots were subculture on hormone free medium for 5 weeks. Data were recorded on rooted shoots, root number, root length and callus formation.

Acclimatization

Plantlets that had fully developed roots were removed from media and rinsed with running water and transferred to greenhouse at 25℃ ±2 with 16/day photoperiod in pots filled with sterile peatmoss: perlite, 3:1: v: v. The plantlets were irrigated with 0.5g/l root enhancer DisperRoot (Manufactured by Eden, Sphenx) and covered with transparent plastic bags to maintain a high humidity level around the plantlets. Three days later tiny holes were performed in the plastic bags, subsequently, the plastic bags were removed after one weeks

RESULTS AND DISCUSSION

Peganum harmala L. is an important folkloric medicinal plant (Aboul-Enein *et al*., 2012). In Egypt, it distributes along Mediterranean cost, Eastern desert and around the ring dyke of Saint Katherine, Sinai. It also binges in the empty spaces, disturbed ground, and near roadways (El-Bakatoushi *et al*., 2011). Harmal plant could be endangered due to the widespread usage of it as a traditional medicine (Saini and Jaiwal, 2000). *In vitro* propagation currently implemented to save endangered plant species. There are relatively limited reports of harmal *in vitro* multiplication therefor, establish-

ment an effective *in vitro* propagation protocol for preservation of *Peganum harmala* L. is the goal of this study. In this investigation the micropropagation protocol was studied using MS medium enriched with BAP, IAA, IBA and NAA. The balance of growth regulators in *in vitro* cultivation depends on the aim, such as shoot or root formation (Legesse and Bekele, 2021). According to Manju *et al*. (2017) the micropropagation phase is referred to as initiation, multiplication and rooting. In the multiplication phase, cytokinin levels should typically be higher than auxin levels. In contrast, during the rooting phase, cytokinin may not always be required and larger doses of auxins may be added to the culture media (Torres- Vazquez *et al*., 2001). To induce multiple shoots in order to establish the effect of BAP preconditioning at the pre-explant stage in relation to multiple shoot induction. Preconditioning BAP treatments is frequently used to improve seed germination and increase seedling vitality (Tiryaki and Ahlatcioglu, 2009). In contrast, during the rooting phase, cytokinin may not always be required and larger doses of auxins may be added to the culture media (Torres- Vazquez *et al*., 2001). Micropropagation system for harmal was adopted through (1) *in vitro* seeds germination and seeds preconditioning (2) formation of viable adventitious shoots from the *in vitro* shoot tip explants and shoots multiplication (3) root development of shoots to form whole plants (4) plantlets acclimatization of developed plantlets .

Seeds germination

Four different media were evaluated for germination. Seeds started to germinate 3 days after culture on all the media contain BAP however, hormone- free medium started after one week. Results showed that GM3 medium containing 1mg/l BAP revealed the highest germination percentage (98%) while, hormone free medium (GM1) gaves the lowest percentage of germination (71.5%). Hyperhydircity was appeared on germinated seeds with a percentage range from 1 to 9% (Table 3) & (Fig.1). Although medium GM1 gave a lowest percentage of hyperhydircity but it was not the highest one for germination. Therefore, medium GM3 was selected as a germination medium of harmal.

Shoot multiplication.

To establish shoot differentiation two explants, i.e., shoot tip (SHT-EX) and cotyledonary segment (CN-EX), grown on different germination media, were cultured on six different media. Shoot formation showed significant difference among treatments, it was ranged from 40% to 100%. The highest shoot formation was revealed with shoot tip explants obtained from GM3 medium and cultured on MM1 medium contains 0.5 mg/l BAP. It's important to note that there

were no growth-related variations between the shoots initiated from the two explants. Hyperhydricity was observed in same cultured in multiplication stage (data not shown).

The percentage of shoot formation; callus formation; number of shoots/explants and shoot length, respectively is demonstrated in Table (4) and Figs (2, 3,4 & 5).

In order to develop an efficient and economical *in vitro* propagation protocol, for rapid and large-scale propagation of harmal plant from shoot apex explant, MS medium supplemented (1.0 mg/l) for the preconditioning stage during seed germination and MS medium supplemented with 0.5 mg/l BAP for the multiplication were the best treatments. In comparison to *Salvia guaranitica* a culture treated with the same amount of TDZ (thidiazuron), media containing 0.5 mg/l benzylaminopurine produced a *Salvia guaranitica* multiplication rate that was 2.5 times higher (Echeverrigaray *et al*., 2010).

It was shown that explants from various germination media responded differently, seedlings grown on 1.0 BAP had the highest proportion of shoots produced, therefore, it was regarded as a precondition for multiplication. Similar to this, a number of plants have been assessed as cultural prerequisites, including *Linnum usitaissium* (Yildiz and Ozgen, 2004; Burbulis *et al*, 2005) and *Vigna mungo* (Saini and Jaiwal, 2007).

Cytokinins play a role in a variety of physiological processes in plants, including the control of seed germination, the growth and proliferation of shoots (Jameson and Song, 2016; Kumar *et al*., 2013). It regulates cell division by promoting a transcription factor that activates mitotic gene expression. cytokinin has a positive-feedback that initiates a transcriptional cascade that drives mitosis and cytokinesis effects in enhancement of cell proliferation and meristem growth (Yang *et al*., 2021). Among the various cytokinins, BAP is the most commonly effective cytokinin for *in vitro* regeneration (Saini and Jaiwal, 2002; Sonia *et al*., 2007) and Chaudhary *et al*., 2007). Addition of BAP during the germination stage has an impact on shoot multiplication (Fig. 1).

On the other hand, the ratio of BAP and NAA during the multiplication stage had an impact on the shoot formation. Similar to Basalma *et al*. (2008), the medium containing 0.5 mg/l BAP + 0.02 mg/l NAA, or 1.0 mg/l BAP + 0.2 mg/l NAA shoot multiplication has experienced significant declines. While, the medium containing 0.5 mg/l BAP alone, in meristem tips exhibited the highest shoot multiplication capacity (100%). Also, tissue-cultured plant material exhibited callus formation and hyperhydricity, as physiological imbalance.

Hyperhydricity, also referred to as ‘vitrification’ or ‘glassiness. It was determined to be the primary cause of the low number of seedlings and shoots. High relative humidity, ethylene content inside

the vessel, and the hormonal make-up are physical and chemical factors that contribute to hyperhydricity. According to prior reports, hyperhydricity impacts production in micropropagation and can result in losses of up to 60% in certain genera. Additionally, the media's inclusion of the cytokinin BAP (to increase the rate of shoot multiplication) adds to the growth of hyperhydric deformities (De Klerk and Martinez, 2010).

In this study, obtained hyperhydrated seedling and shoots in this investigation might be due to high level of the growth regulators BAP and NAA on multiplication and high BAP on germination medium. Hyperhydricity was overcome by transferring them to fresh medium such as *Plumbago rosea* (Harikrishan and Hariharan, 1996), *Alinia galangal* (Anand and Hariharan, 1997), *Vigna mungo* (Saini and Jaiwal, 2002), *Vigna radiata* (Sonia *et al.*, 2007). The effects of varied concentrations of BAP and NAA on various stages of tissue culture were dependent on both the individual and combined concentrations. The formation of calluses was often accelerated if NAA and BAP were in equilibrium (NAA/BAP).Media supplemented with more concentration of BAP with no or low levels of NAA stimulate shoot formation and reverse media type-initiated roots. On media treated with 0.5-0.1mg l of BAP and NAA, respectively, maximum callus development was seen, but the highest regeneration was shown on media supplied with 0.5 mg l of BAP only such as (Ahmad and Spoor, 1999).

Root formation and adaptation

Generated roots were obtained during five weeks. Only shoots on full MS media with varying IAA concentrations were rooted during the first two weeks; however, hormone-free medium and media with varying NAA or IBA concentrations failed to generate any roots during that time. Furthermore, NAA media promote callus induction (Fig. 6). It was observed that the first roots appeared 10 days after cultivation. A percentage of 13%, 6.5%, and 6.5% the roots were developed on media with 0.5, 1, and 1.5 IAA, respectively. After subculturing on hormone-free MS medium shoots from 0.5 IAA were produced more roots with a total percentage of 84% within a week to three weeks, while, shoots from other concentrations failed to produce any roots (Fig. 7). Therefore, 0.5 IAA medium was chosen as the optimal medium, then subculturing on hormone-free MS medium for root development. Rooted shoots were successfully adapted under greenhouse condition. Fig. (8) demonstrates harmal, micropropagation steps.

Adventitious root development on micro shoots is a crucial stage, to obtain plantlets that are ready to be acclimated to *ex vitro* environments. Harmal microshoots were rooted more successfully on medium containing 0.5 mg/l (IAA). The highest root production (13%), in comparison with full or half MS strength medium supplemented with IBA (0;0.5;1;1.5 mg/l), IAA (0; 1;1.5 mg/l) or NAA (0;0.5;1;1.5 mg/l). NAA or IBA concen-

trations failed to generate any roots. Unlike, Çetin and Koçak (2020) IBA were made to root the obtained shoots of harmal. Also, Zatimeh *et al*. (2017) showed that NAA (1mg/l) used to root the obtained shoots of harmal. In addition, they found that supplementation of rooting medium with IAA (1;1.5 mg/l) the rooting percentage was 6.5%. The obtained root formation efficiency in the different rooting cultures under study which was 6.5-13% would not be sufficient for massive production of harmal.

Rooting limitations severely hinder the species' ability to reproduce and also create a problem for its commercial development (Wiszniewska *et al*., 2016). In this study, rooting was also enhanced to (84%) after subculturing on MS free hormone free medium. The highest root formation, were increased threefold after subculturing on hormone comparison with induction medium containing IAA. The best root formation was observed on the 0.5mg/l of IAA/free hormone MS media. However, it was previously noticed that as the exogenous IBA concentration in the culture medium was raised further (to more than 0.5mg/l), root induction gradually decreased. It's interesting that the hormone-free media formed roots on microshoots, maybe because endogenous auxins were present similar to (Amiri and Mohammadi, 2021).

According to this study, IAA was the only hormone promoted root development in among the tested hormones. In contrary of our results, Musaallam *et al*. (2011) stated that NAA poor root production while it was high on media containing IAA. Acclimatization was successful when plantlets were transferred into greenhouse.

The results of this study mark a positive development for the conservation of plant genetic resources as well as the *in vitro* cloning of valuable genotypes directly from field-grown plant

SUMMARY

Since ancient times, medicinal plants have been essential to human civilization, and they are still essential now. *Peganum harmala* L. regarded as one of the most significant medicinal plants. This perennial herb is indigenous to Central Asia and the Mediterranean region and is a member of the Nitraraiceae family. *Peganum harmala* L. is widely distributed in Egypt along the left bank of the Mediterranean, in Sinai, the Eastern Desert, and in open areas with disturbed ground. Recent reports indicate that this plant contain beta-carbolines alkaloids such as harmaline, harmine and harmalol therefore, it has antibacterial, antifungal, anti-inflammatory and anti-cancer properties. The main challenges to the sustainable propagation and supply of this valuable herb are the short seed viability period and unrestricted large collection. In this study for multiple shoot induction and plant regeneration of *Peganum harmala* L. has been successfully developed using shoot apex and cotyledonary node explants. This study also demonstrates that preconditioning of explant stimulates

production of multiple shoots from shoot apex explant were excised from seedlings germinated on Murashige and Skoog (MS) media supplemented with benzylamino purine (BAP)1.0 mg/l and subsequently cultured on MS media with 0.5 mg/l BAP exhibited higher multiple shoot development than the cotyledonary explants, which, produced 8.1 shoots/explant (93.3%) and had no hyperhydricity among all treatments.

After cultivation on MS with 0.5 mg/l IAA followed by cultivation on hormone-free MS medium, roots were produced with an efficiency of (84%). Rooted plantlets were successfully acclimatized at the greenhouse conditions (25±2 °C).

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Table (1): Different germination media composition.

Germination medium (GM)	Media composition
GM1	MS
GM2	MS,0.5 mg/L BAP
GM3	MS,1.0 mg/L BAP
GM4	MS,2.5 mg/L BAP

Table (2): Different multiplication media composition.

Multiplication media (MM)	Media composition	
	BAP mg/l	NAA mg/l
MM1	0.5	0
MM2	1.0	0
MM3	0.5	0.02
MM4	1.0	0.02
MM5	0.5	0.1
MM6	1.0	0.1

Table (3): Seeds germination percentage on different media composition.

Germination media	Germination percentage	Hyperhydircity percentage
GM1	71.5%	1%
GM2	73%	4.5%
GM3	98%	1.5%
GM4	83%	9%

Table (4): Shoot formation from shoot tip & cotyledonary node explants.of *Peganum harmala* on different media composition.

Seedling source	Multiplication media	Shoot formation (%)		Callus formation (%)		Mean of number of shoots per explant		Shoot length	
	MM1	shoot tip	cotyledonary node	shoot tip	cotyledonary node	shoot tip	cotyledonary node	shoot tip	cotyledonary node
		80	53	0	0	3.8	3.55	2.5	3
GM1	MM2	86	100	0	0	4.1	4.8	2.5	3
	MM3	80	86.6	0	0	3.6	5	2.5	2.5
	MM4	53.3	100	0	26.6	2.8	4.6	2.5	2.5
	MM5	53	80	0	60.6	3.3	5.7	3	3
	MM6	73	93.3	0	53.3	1.1	4.6	3	2.5
GM2	MM1	40	53	0	0	3.5	3.55	2	3
	MM2	93	100	0	0	3.6	4.8	3	3
	MM3	100	86.6	0	0	3.6	5	2.5	2.5
	MM4	93.3	100	13.3	26.6	3.2	4.6	2	2.5
	MM5	86.6	80	60	60.6	2.1	5.7	1.5	3
	MM6	93.3	93.3	53.3	53.3	3	4.6	3	2.5
GM3	MM1	93.3	93.3	0	0	8.1	4.5	5	5
	MM2	73.3	86.6	0	0	2.9	3.3	2.5	2.5
	MM3	73.3	80	0	20	2.6	4.5	3	3.5
	MM4	80	86.6	13.3	13.3	3.1	5	2.5	4
	MM5	80	100	40	26.6	3.8	7	4.5	3
	MM6	93.3	93.3	46.6	46.6	5.8	6.1	3	3
GM4	MM1	100	93.3	0		7.6	4.6	5	4
	MM2	100	100	6.6	6.6	8	5.5	2.5	4
	MM3	86.6	100	13.3	13.3	3.75	5.2	3.5	3.5
	MM4	93.3	93.3	13.3	20	3.2	7	2	2.5
	MM5	73.3	100	26.6	33.3	3.6	5.5	2.5	3
	MM6	93.3	86.6	40	40	6.1	5.1	4	3.5

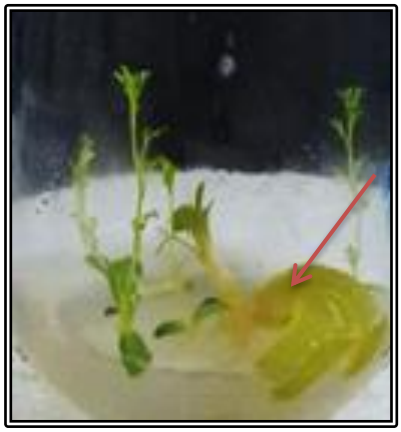
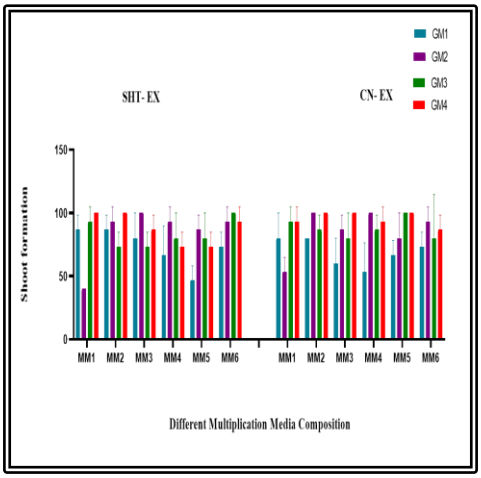
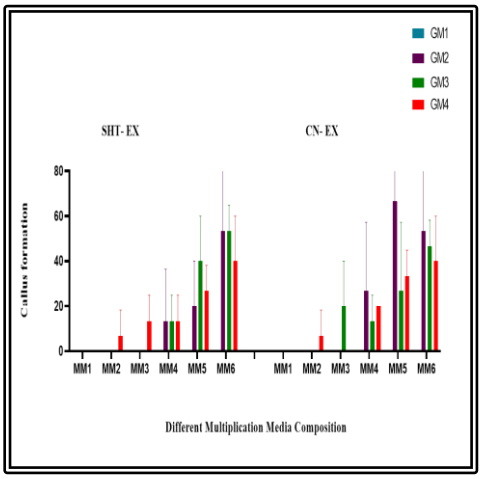


Fig.(1):: Seed germination stage: the red arrow demonstrates the hyperhydrated seedling.



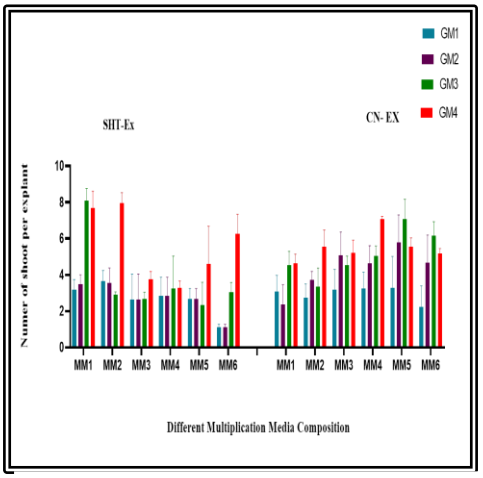
**** Significant P value ≤0.0001

Fig. (2): Shoot formation percentage of *Peganum harmala* on different media composition.



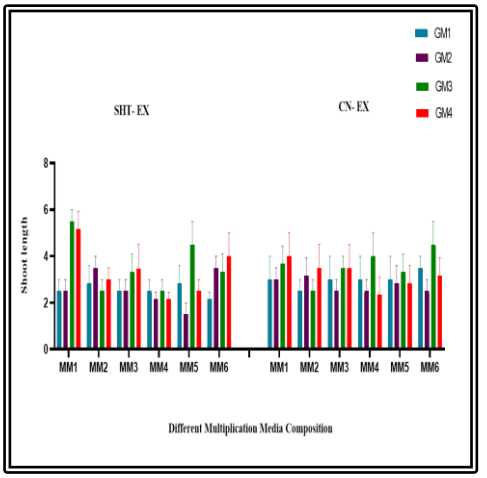
*** Significant P value ≤0.0002

Fig. (3): Callus formation percentage of *Peganum harmala* on different media composition.



**** Significant P value ≤0.0001

Fig. (4): Number of shoots/explants of *Peganum harmala* on different media composition.



**** Significant P value ≤0.0001

Fig. (5): Shoot length of *Peganum harmala* on different media composition.

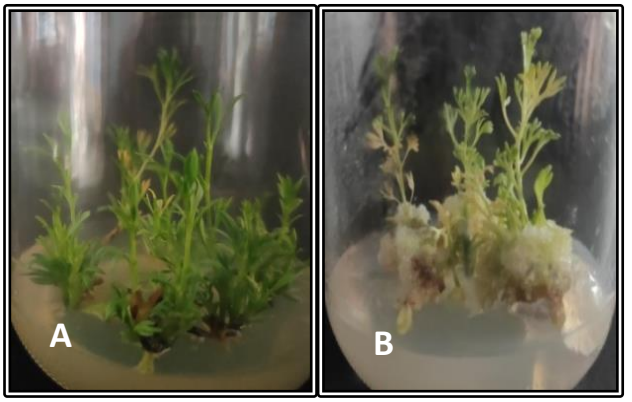


Fig (6): (A) Shoots with root on .05 IAA, (B) Callus formation on shoots at NAA

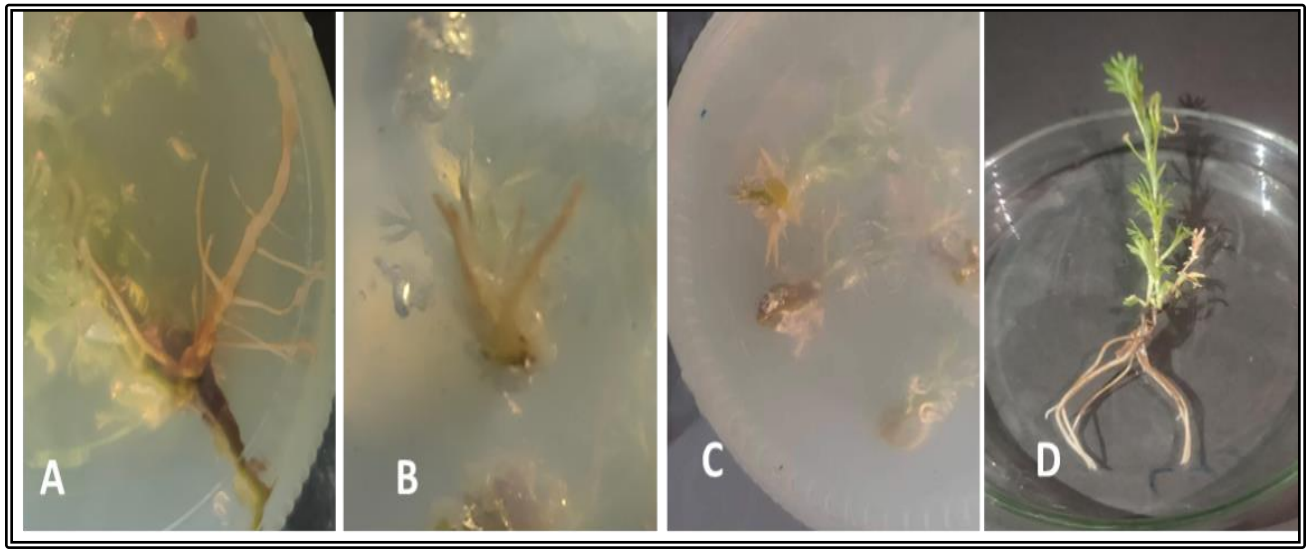


Fig 7: Roots on different IAA concentration A) 0.5, B) 1.0 & C) 1.5; D) root formation after culturing on hormone free medium.

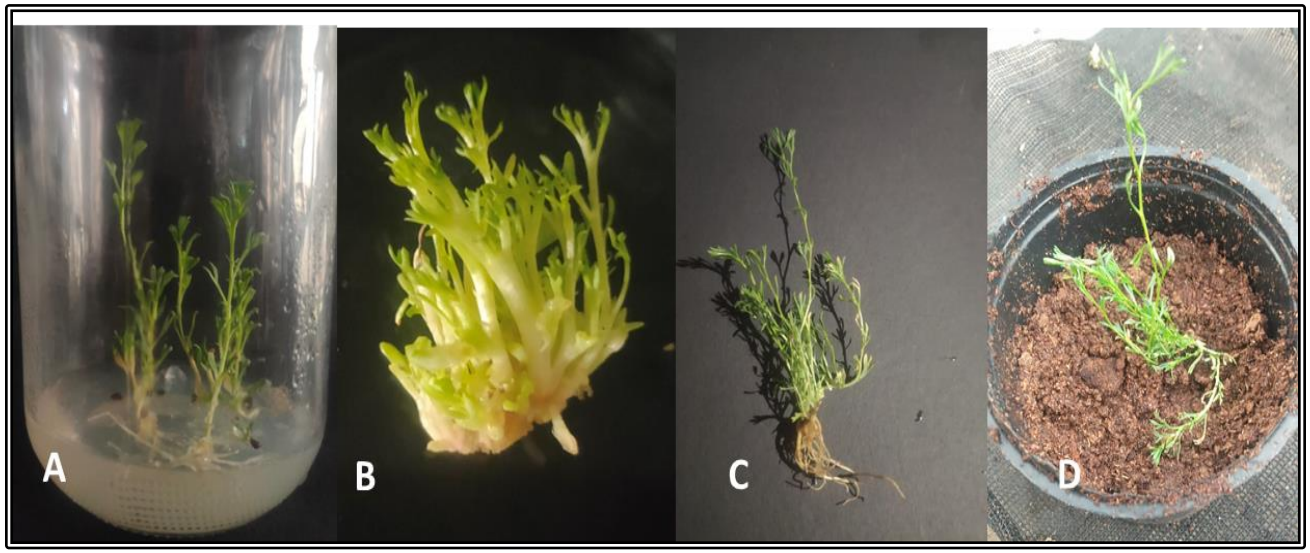


Fig.(8): Micropropagation steps of *Harmal*, A) Seed germination on 1.0 mg/l BAP after 15 days; B) Shoot formation after 3 weeks on MS medium supplemented with 0.5 mg/l BAP; C) Root formation on 0.5 IAA, hormone free media after 5 weeks and D) acclimatized plant under greenhouse condition.