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Original article

Micropropagation of the Endangered Medicinal Plant Thymus decussatus

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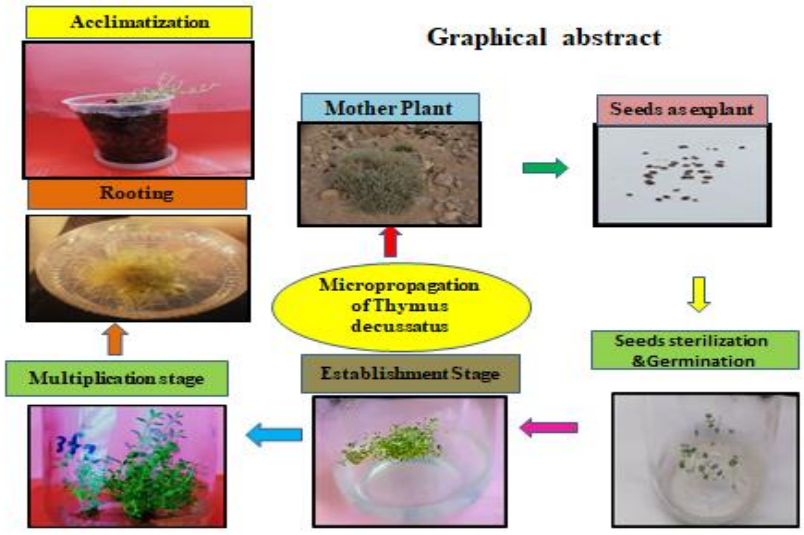
ABSTRACT

Thymus decussatus is a perennial herbaceous endangered medicinal plant found on mountain peaks in Egypt's Saint Katherine Protectorate. A strategy for *in vitro* preservation of this rare and a valuable medicinal plant was developed using Micropropagation technique. For the best shoot development, stem segment explants from one-month-old in vitro germinated seedlings were cultured on Murashige and Skoog's medium (MS media), which was supplemented with kinetin (Kin; 1.00, 2.00, and 3.00 mgl-1) and 6-benzylaminopurine (PAB; 0.50, 1.00, and 1.50 mgl-1) in various concentrations. After 4-5 weeks, MS medium with 1.00 mgl-1 KIN exhibited the highest value for shoot formation. Naphthalene acetic acid (NAA) and 3-indole acetic acid (IAA) were added to MS medium in various concentrations (0.50, 1.00, 1.50, and 2.00 mgl-1) to promote the development of roots. The greatest root numbers were attained on MS medium with 1.00 mgl-1 NAA after 5–6 weeks. Plantlets with strong roots were effectively acclimated, surviving at a rate of 73%.

Graphical abstract



1. Introduction

There are 928 species in the genus Thymus, which is a member of the Lamiaceae family, and 215 of them are mostly found in the Mediterranean area [14], [29]. The thyme plant has antibacterial, antiparasitic, antispasmodic, and antioxidant capabilities because of its fragrant character [25, 27]. The Thymus genus is well-known for treating nausea and has a number of traditional uses against a wide

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range of illnesses, including headache, ulcers, eczema, renal problems, asthenia, wounds, verrucae, and diabetes [17]. Sinai, Egypt is home to the incredibly uncommon plant T. decussatus Benth. [8]. There have been a few investigations on the chemical composition and biological properties of T. decussatus essential oil (EO), including cytotoxicity and antibacterial activity [3, 10]. It has become one of the most significant and often used medicinal herbs as a consequence of its therapeutic characteristics [22].

Due to human activities, conventional food supplements, overharvesting for fuel and pharmaceutical health products, a constrained range distribution, a low rate of natural reproduction, overexploitation of mines, and persistent overgrazing in recent decades, T. decussatus is on the verge of extinction and is categorized as an extremely vulnerable species in Egypt [15]. In order to restore very deprived populations, a more effective regeneration method for conservation and multiplication is clearly needed. There are several benefits to in vitro propagating endangered plants, including the capacity to quickly produce species with poor reproductive potential and those that live in vulnerable ecosystems [11]. The maintenance of uncommon and endangered plant species is becoming more and more dependent on in vitro propagation techniques, which are also necessary for managing plant genetic resources. [26].

Focusing on the in vitro propagation of Thymus vulgaris, [12] the in vitro cultures were started with apical and axillary buds acquired from field-grown plants and nodal segments multiplied on semi-solid Nitsch and Nitsch media [23] containing kinetin and indole 3-butyric acid (IBA) or NAA. In other papers, procedures for Thymus mastichina L., T. sipyleus Bioss., and T. piperella are described as micropropagation or organogenesis. **[6]** demonstrated that the growth regulators utilized and the source of the explants had an impact on how well plants regenerate. Your research also showed that Thymus persicus explants behave differently depending on the growth regulators added to the culture media. **[16]** said that Thymus vulgaris L. may be propagated in vitro by starting in vitro cultures from shoot explants and growing them on MS media supplemented with BAP, 2iP, or KIN. Their findings showed that 5mgl dm3 2iP had the greatest beneficial effect on plant development during the multiplication stage. The best rooting of shoots was achieved using MS medium and 2 mg/dm3 BAP.

2. Materials and methods

Gathering and sterilizing plant material

Seeds of *T. decussatus* plant were obtained from wild populations in the Saint Katherine Protectorate's Mountain tops (Figure 1 A, B). Detachment from the mother plant was followed by an hour of rinsing under running water, followed by 30 minutes of pre-treatment with liquid detergent. After cleaning the seeds, 70% ethanol was applied for 30 seconds. The seeds were then washed with sterile distilled water, soaked in varying percentages of 5% sodium hypochlorite (NaOCl) for 10, 12, and 15% of the time, and then rinsed four or five times with sterile double distilled water. The sterilized seeds were then grown on MS medium five duplicates of each treatment were placed in 250 ml glass jars with 50 ml of nutritional media (10 seeds per jar), which were then firmly closed with plastic lids. The culture was maintained in a growth chamber with a 25°C temperature and a 16–8 hours photoperiod powered by white fluorescent lights. Ten days after culture, the proportion of seeds that perished or were infected was determined.

Establishment and multiplication of *T. decussatus in vitro*:

Stem segment explants from one-month-old in vitro germinating seedlings were excised and transplanted to MS baseline media supplemented with several cytokinin types, including 6-benzylaminopurine (BAP) and kinetin (KIN), both at concentrations of 1mgl-1, in order to accelerate shoot formation. Explants produced during the establishment stage were used for the induction of numerous shoots. All explants were grown in MS medium that also contained BAP and KIN in varying amounts (0.50, 1.50, and 3.0 mgl-1).

Figure 1: *Thymus decussatus* **A.** wild plant of *T. decussatus* **B**. seeds of the plant

In vitro effects of BAP and KIN as cytokinin on shoot establishment of *T. decussatus* explants

Cytokinins	Conc. (mgl^{-1})	Mean number of shoots/explant	Average shoot length in cm
Control	0.00	7.00	0.98
BAP	1.00	6.00	0.95
KIN	1.00	10.67	1.44

In vitro effect of various KIN and BAP concentrations on multiple shoot induction from *T. decussatus* stem segment explants

Cytokinins	Conc. (mgl^{-1})	Mean no. of shoots/ explant	Average shoot length in cm	Mean number of leaves per transplant
Control	0.00	7.25	0.98	113.00
	0.50	14.33	2.11	158.00
BAP	1.00	8.67	1.26	89.70
	1.50	6.00	1.19	56.33
	1.00	**38.33**	**2.65**	**185.30**
KIN	2.00	12.33	1.92	111.7
	3.00	9.33	1.65	102.33

Taking root and acclimatizing

The resulting elongated shoots were then transplanted into MS basal medium fortified with various concentrations of 3-indole acetic acid (IAA) and naphthalene acetic acid (NAA) alone (0.50, 1.00, 1.50, and 2.00 mgl-1), as well as free MS medium without plant growth regulators as a control. After five weeks, roots' numbers, lengths, and thicknesses were measured. Plantlets with established roots were removed from the culture medium, carefully washed with distilled water to remove any remaining material, and then placed into plastic pots filled with sterile media comprised of sand and peat moss in a 1:1 ratio. According to [20], the cultures were covered with a thin layer of plastic sheet and irrigated with sterile tap water.

They were then housed in the same circumstances in a growth chamber. After three weeks, the plastic sheets were gradually removed, and the rooted plantlet were gently transplanted into larger pots and kept in a greenhouse environment.

Analytical statistics

Data from all experiments were subjected to one-way analysis of variance (ANOVA) in the (Minitab 19) system using the general linear models (GLMs) method. For mean comparisons, the least significant difference (LSD) method was applied.

3. Results and discussion

All seeds cultured on full strength MS medium after sterilization treatments (10 seeds / jar) with five replicates.

Surface sterilization

The choice of sterilization techniques relies on how frequently contamination and mortality occur. High sterilizing agent concentration and duration prevent contamination but increase the death rate and vice versa. The data in table (1) illustrate the influence of different NaOCl solution concentrations for varied durations on the survival percentage of *T. decussatus* seeds.

The highest survival rate (66%) was obtained after the treatment with 12% NaOCl for 15 min **(Figure 2).** It can be observed that, increasing the period of sterilization, reduced the percentages of death and contamination. The same treatment after 10 minutes resulted in 48% survival rate while the same concentration after 20 minutes resulted in 40% survival rate.

The results showed that, NaOCl at the appropriate concentration is a suitable sterilizing agent with a high seeds survival rate and low contamination, which confirms previous studies on various plant species such as *Thymus persicus* **[6],[7];** *Teucrium polium* L. **[5];** *Zataria multiflora* **[21]** and *Thymus sibthorpii* **[21]**. In contrast, **[21]** demonstrated that 0.1% (w/v) mercuric chloride ($HgCl_2$) is an efficient regimen for sterilizing explants intended for micropropagation of valuable lamiaceae species.

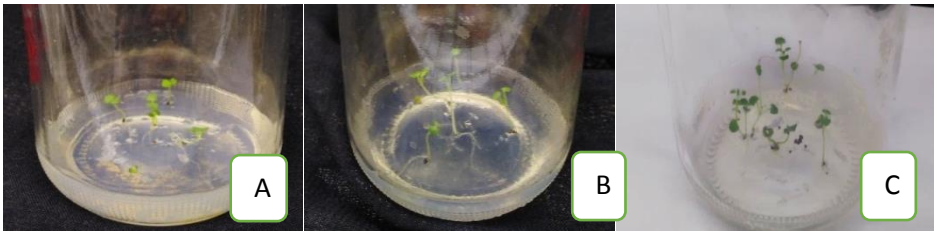

Figure 2: A, B Seeds germination after 10 days of culture on free MS medium treated with 12% of NaOCl for 15 min, **C** germination after 21 days

Table (1) Effect of different sterilization treatments with different concentrations of sodium hypochlorite solution for various duration on the survival of *T. decussatus* seeds.

Sterilization treatments	Mean % of survival	Mean% of contamination	Mean% of death
10% of NaOCl - 10 min.	34 ± 6^{bcde}	42 ± 5.83^{a}	24 ± 2.45^{e}
10% of NaOCl - 15 min.	30 ± 3.16^{bcde}	30 ± 4.47^{ab}	40 ± 3.16^{cde}
10% of NaOCl - 20 min.	24 ± 6.78^{cde}	20 ± 9.49^{abc}	56 ± 4^{bcd}
12% of NaOCl - 10 min.	48 ± 3.74^{abc}	10 ± 4.47^{bc}	42 ± 7.35^{cde}
12% of NaOCl - 15 min.	66 ± 4^{a}	0 ± 0^{c}	34 ± 4^{de}
12% of NaOCl - 20 min.	40 ± 5.48^{bcd}	0 ± 0^{c}	60 ± 5.48^{abc}
15% of NaOCl - 10 min.	52 ± 5.83^{ab}	10 ± 6.32^{bc}	38 ± 3.74^{cde}
15% of NaOCl - 15 min.	18 ± 4.9^{de}	10 ± 4.47^{bc}	72 ± 3.74^{ab}
15% of NaOCl - 20 min.	12 ± 8^{e}	6 ± 4^{bc}	82 ± 6.63^{a}

Each value represents Mean ± SE. Means that do not share a letter are significantly different. Results recorded after 10 days of culture.

Establishment stage

For this stage, stem segments excised from one month old seedlings were used as the explant source for shoot induction. The mean number of shoots generated per explant and mean shoot length were determined after four weeks of culture. MS medium has been identified as the preferred medium for *in vitro* cuture initiation **[1],[13].**

The information in table (2) and figure (3) shows that there is a substantial relationship between the kind of cytokinin and the average number of shoots and shoot length. The MS medium supplemented with 1.0 mgl-1 KIN generated the most multiple shoots per explant (10.67), with a mean length of 1.44cm, when compared to the other media. This outcome is in line with what was seen by [24], who noted that Thymus vulgaris and T. longicaulis produced numerous shoots at the maximum rate on MS medium supplemented with 1.00 mgl-1 KIN. Additionally, name of author [1] noted that for Origanum syriacum, 1.00 mgl-1 KIN generated the most shoots per explant. Name of author [28] discovered that Mentha piperita regenerated best and multiplied optimally when grown on MS medium supplemented with 2.32 M KIN. However, according to name of author [18], Thymus moroderi is a cytokinin-sensitive species with low concentrations that negatively affects in vitro germinating plants. Furthermore, it was discovered that Thymus lotocephalus shoot growth was most favored by greater BAP concentrations (>2.22 M) [9].

Table 2.'' Effect of BAP and KIN as cytokinin on shoot establishment from *T. decussatus* explants *in vitro''*

Plant growth control agents	Conc. (mgl^{-1})	Mean number of transplanted shoots ±SE	St. Dev	Mean shoot length (cm) ± SE	St. Dev
Control	0.00	7 ± 1.53^{ab}	2.65	0.98 ± 0.30^{b}	0.60
BAP	1.00	6 ± 2.52^{b}	4.36	0.95 ± 0.12^{b}	0.28
KIN	**1.00**	$\mathbf{10.67\pm0.88^{a}}$	**1.53**	$\mathbf{1.44 \pm 0.28^{a}}$	**0.57**

Each value represents Mean±SE. Means that do not share a letter are significantly different. Results recorded after 4 weeks of culture.

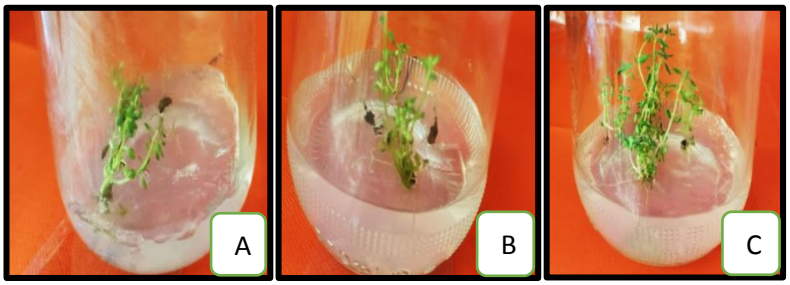

Figure 3. '' T. *decussatus* establishment in vitro: After around 4 weeks, stem segments were established on A. basic MS medium (control), B. MS medium supplemented with 1.00 mg l-1 BAP, and C. MS medium supplemented with 1.00 mg l-1 KIN**.''**

Multiplication stage

Multiplication is a rapid increase of organs that can eventually give a rise to plants. This is accomplished by increasing axillary shoot initiation. Shoot proliferation *in vitro* is affected by the type and concentration of plant growth regulators (PGRs) as

recorded by name of author **[2]. Table 3** shows the response of stem segment explants to various concentration of cytokinin employed for shoot multiplication. The development of shoots was considerably impacted by various concentrations of BAP and KIN. The highest rate of multiplication was obtained from stem segment explants maintained on MS media supplemented with 1.00 mgl^{-1} KIN **(Figure 4),** resulting in 38.33 shoots/explant, 2.65 cm with 185.30 leaves/explant**,** and this medium was chosen as the best medium for shoot multiplication. This was followed by 0.50 mgl^{-1} BAP, which resulted in 14.33 shoots/explant, 2.11 cm with 158 leaves/explant. The results of this study accord with those of name of authors **[24]** and **[4].**

Table 3. ‘’ Effect of various BAP and KIN concentrations on the in vitro proliferation of T. *decussatus* stem segment explants*”*

cytokinin	Conc. (mgl^{-1})	Mean no. of shoots/explant (no. ± SE)	Mean shoot length (cm ± SE)	Mean no. of leaves /explant
Control	0.00	7.25 ± 1.49^{b}	0.98 ± 0.30^{b}	113 ± 29.1^{ab}
	0.50	14.33 ± 0.88^{ab}	2.11 ± 0.08^{a}	158 ± 9.54^{ab}
BAP	1.00	8.67 ± 2.4^{b}	1.26 ± 0.19^{b}	89.7 ± 31.9^{b}
	1.50	6 ± 2.52^{b}	1.19 ± 0.05^{b}	56.33 ± 7.75^{b}
	1.00	38.33 ± 5.81^{a}	2.65 ± 1.08^{a}	185.3 ± 38.1^{a}
KIN	2.00	12.33 ± 0.88^{ab}	1.92 ± 0.39^{ab}	111.7 ± 17.4^{ab}
	3.00	9.33 ± 0.88^{b}	1.65 ± 0.26^{ab}	102.33 ± 3.33^{ab}

Each value represents Mean ± SE. Means that do not share a letter are significantly different. Results recorded after 5 weeks of culture.

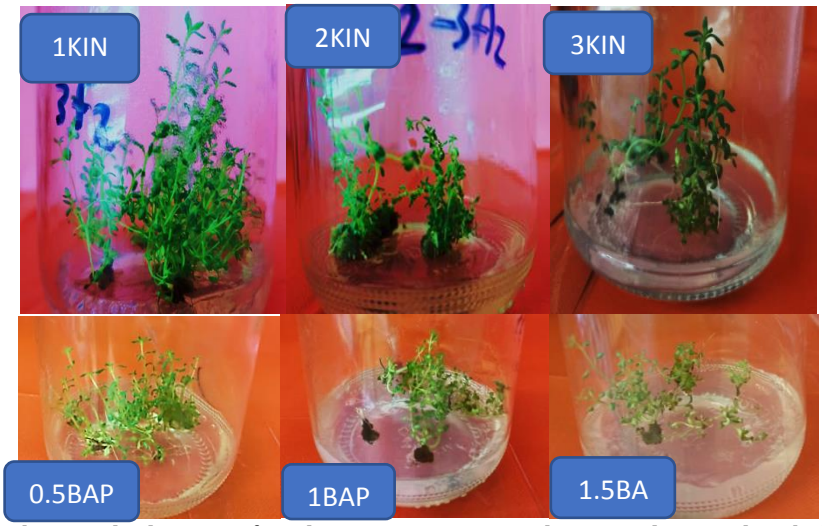

Figure 4. Shoot multiplication of *T. decussatus* on MS medium supplemented with different concentrations of KIN (1.00, 2.00 and 3.00 mgl^{-1}) and BAP (0.50, 1.00 and 1.50 mgl^{-1}) after 5 weeks.

The results showed that increasing in KIN and BAP concentrations resulted in a decrease in the mean number of shoots, shoot lengths and leaves number generated per explant **(Figure 4)**. Thus it can be concluded that the maximum multiplication rate of *T. decussatus* can be obtained on MS medium fortified with 1.00 mgl^{-1} KIN after 5 weeks.

Rooting stage

Well-developed shoots from the previous stage were transferred to MS medium with or without varying auxin concentrations (IAA, NAA) ranging from 0.00 to 2.00 mgl^{-1} for root formation. After 5 weeks of culture, the influence of auxins on root induction was investigated. The presented data in **table 4** show that full strength MS medium supplemented with 1.50 mgl^{-1} IAA followed with 1.00 mgl^{-1} NAA was found to be the most effective for root length, while full strength MS medium supplemented with 1.00 mgl^{-1} NAA followed with 1.50 mgl^{-1} IAA was found to be the most effective for root numbers. The highest mean root numbers were 35 and 31.75 on MS medium enriched with 1.00 mgl^{-1} NAA and 1.50 mgl^{-1} IAA, the maximum mean root length was 1.17 cm was obtained on MS medium containing 1.50 mgl^{-1} IAA **(Figure 5)**. NAA containing media produced short, thick roots, some of which were callogenic at certain concentrations, whereas media containing IAA and medium devoid of auxins (control) generated long thin roots. These results are consistent with those obtained by **[6].** According to the results, the greatest percentage of *T. decussatus* shoots that developed roots was achieved after 5 weeks on MS medium fortified with 1.50 mgl^{-1} IAA and 1.00 mgl^{-1} NAA. These findings are consistent with those found by **[5].** Previous research found that MS medium supplement-

ed with indole 3-butyric acid (IBA) was more effective for root formation *Origanum syriacum* L., **[31]**;*T. sibthorpii* Benth. **[6].**

Table 4. Effect of MS media supplemented with various auxin concentrations on the development of roots from T. *decussatus* shoots that have been in vitro regenerated

Plant growth regulators	Conc. (mgl^{-1})	Mean no. of roots (no. ± SE)	Mean root length (cm ± SE)	%of rooted shootlet	Root morphology
Control	0.00	5.50 ± 1.94^{a}	0.83 ± 0.05^{ab}	25^{d}	Thin, long
IAA	0.50	19.75 ± 3.15^{ab}	0.31 ± 0.07 ^{b}	66.67^{bc}	Thin, long
	1.00	25.25 ± 8.87 ^{ab}	1.03 ± 0.15 ^{a}	91.67^{ab}	Fragile, long
	1.50	**31.75 ± 6.84 ^{a}**	**1.17 ± 0.197 ^{a}**	**100^{a}**	**Fragile, long**
	2.00	11.33 ± 5.24 ^{b}	0.18 ± 0.05 ^{b}	25^{d}	near the base, a thick, short callus
NAA	0.50	21 ± 9.54 ^{ab}	0.53 ± 0.09 ^{ab}	41.67^{cd}	thick, brief
	1.00	**35 ± 9.41 ^{a}**	**0.84 ± 0.23 ^{ab}**	**100^{a}**	near the base, a thick, short callus
	1.50	15.75 ± 8.23 ^{b}	0.26 ± 0.19 ^{b}	66.67^{bc}	near the base, a thick, short callus
	2.00	*	*	*	*

Each value represents Mean ± SE. Means that do not share a letter are significantly different. Results recorded after 5 weeks of culture.

Acclimatization

Healthy plantlets with strong roots were placed in plastic pots with sterile medium made of sand and peat moss in a 1: 1 ratio for acclimation. It was discovered that T. decussatus plants grown in vitro needed to gradually acclimate to their surroundings. After 4-5 weeks in soil, 73% of the seedlings that were transplanted to pots continued to thrive (Figure 6). The regenerated plants grown in the greenhouse had no morphological variations. Salvia [30] and Thymus persicus [6] have shown similar results.

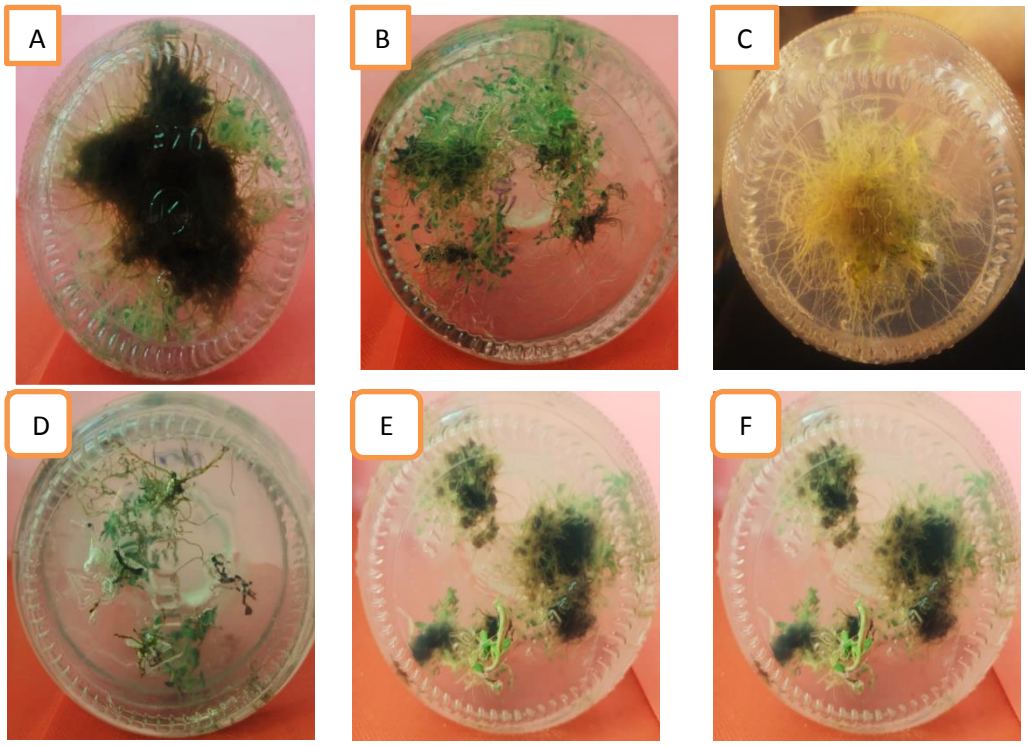

Figure 5. Root formation with high root numbers. **A.** Rooting on MS medium with 1.00 mg l^{-1} NAA. **B**. Rooting on MS medium with 1.00 mg l^{-1} IAA**. C.** Rooting formation with high root length on MS medium with 1.50 mg l^{-1} IAA. **D.** Rooting on free MS medium (control). **E.** Rooting on MS medium with 1.00 mg l^{1} NAA. **F**. Rooting on MS medium with 2.00 mg l^{-1}IAA after 2 months.

4. Conclusion

It may be said that KIN was more successful at inducing shoots and multiplying T. decussatus stem segment explants in vitro. The auxin that had the greatest impact on root development was IAA. An effective method for T. decussatus direct in vitro multiplication is described in the current work. T. decussatus is a natural source of the triterpenes that have antibacterial, antiparasitic, antispasmodic, and antioxidant properties.

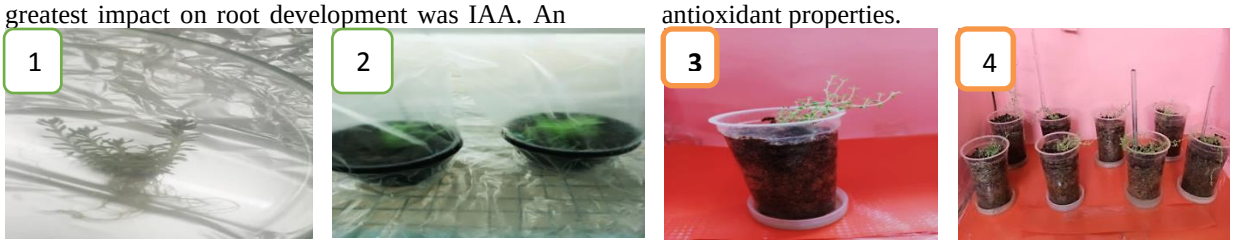

Figure 6: Plantlets with well-developed roots acclimating

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