Micropropagation of Different Species of Thymus

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ABSTRACT

In this work in-*vitro* propagation of Thymus species has been obtained from different explants. In vitro propagation of *Thymus mastichina* L. has been obtained from mature field grown plants. Nodal segments were cultured on MS modified medium supplemented with 0.1mg/l Benzyl amine purine (BAP). The rooting percentage (root number, longest root length and shoot length) were observed with 1mg/l Naphthalene acetic acid (NAA). For *Thymus vulgaris* apical meristem were cultured on MS 1962 medium supplemented with 4mg/l BAP. In In-vitro propagation of *Thymus sipyleus*, the plantlets were regenerated from seedlings which were transferred to MS basal medium containing 0.4mg/l NAA & 3mg/l BA and then they formed callus when in contact with medium. Plantlets which regenerated on the same medium formed multiple shoots within 2 weeks and they were rooted after the 4th subculture.

Key words: Aromatic plants, micropropagation, thyme, *Thymus vulgaris, Thymus sipyleus, Thymus mastichina, Thymus piprella*

INTRODUCTION

Thyme is a semi-woody sub shrub with aromatic, linear to oval, slightly to mentose (fuzzy), gray-green leaves that is about a half inch long. It is an aromatic shrub distributed in the temperate zone, chiefly in the Mediterranean region. The herb has a pungent taste. Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals and cosmetics (Simon *et al.,* 1999; Senatore, 1996). It is also an herbal medicine used as hyper anemic, antibacterial, deodorizing agent in inflammation of the mouth and throat, a diuretic, urinary disinfectant and

vermifuge (*Bisset, 1994*). Traditionally basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms and kidney malfunction (Simon *et al*., 1999). Essential oils from thyme *(Thymus vulgaris L.)* have antioxidant properties, which may result from the presence of free radical scavengers in these oils (Farag *et al*., 1989 ; Deighton *et al*., 1993; Aeschbach *et al*., 1994). In addition, these compounds have been shown to have bactericidal and fungicidal properties (Beuchat, 1976; Conner and Beuchat, 1984; Akgul and Kiranc, 1988; Sharpiro *et al*., 1994; Curtis *et al*., 1996). Thymol has antispasmodic, antibacterial, antifungal, antiseptic as well as antiviral activities (Grieve, 1984). This Herb is also used in making soaps, perfumes, garnings, pastes etc. It is used in both Homeopathic and Allopathic medicine. In early pharmacological works, the extracts of thyme were reported to show antioxidant activity (Economou *et al.,*1991; Schwarz *et al*.,1996) estrogen and progestin bioactivity (Mendes and Romano, 2004), and antimutagenic activity (Miura and Nakatani, 1989). Some methylated flavones were isolated from thyme and tested as antioxidants (Murashige, and Skoog, 1962); three biphenyl compounds were identified as deodorant compounds (Nakatani, *et al*., 1989) luteolin was identified to be as strong anti mutagen against Trp-2, one of the dietary carcinogens formed during cooking (Prasad, 1999), two compounds 3, 4, 3, 4-tetrahydroxy-5, 5-diisopropyl-2, 2-dimethylbipheny and eriodicytol were found to protect biological systems against various oxidative stress.

MATERIAL AND METHODS

Explants of thyme were collected from the gene bank. The explants were surface sterilized in a 20% (v/v) sodium hypo chloride solution containing 0.04% tween 20 for 30 min; followed by three rinses in sterile distilled water in a laminar flow hood. For callus induction, explants consisting of nodal segment and apical meristems were cultured in the MS medium. The pH was adjusted to 5.8 before autoclaving at 120ºC & 15lb pressure for 20-25min. The effect of growth regulators were tested using 2, 4-D (2mg/l) in combination with Kn (0.5mg/l) or using NAA (0.4mg/l) in combination with BAP (3mg/l).). Medium with out plant growth regulators was used as a control. To induce and proliferate shoots, the apical meristem taken from the gene field were transferred to shoot induction medium containing MS supplemented with 4mg/l BAP, 3% sucrose

and pH 5.8. Explants were kept under the dark conditions for 2 weeks and were transferred to the light. For root induction, elongated shoots (14cm) were isolated and transferred to MS medium supplemented with 1.1mg/l BAP and MS with 0.3mg/l IBA + 3mg/l BAP. For *Thymus sipyleus* sterilized seeds of the plant were germinated on modified Murashige & Skoog medium (mMS) and Hellers nutrient media. Then, seedlings were transferred to Murashige & Skoog basal medium containing 0.4 mg/l Naphthalene acetic acid (NAA) and 3 mg/l Benzyl adenine (BA) and then they formed callus when they were in contact with the medium. Plantlets which regenerated on the same medium formed multiple shoots within 2 weeks and they were rooted after the 4th subculture. For *Thymus piperella* explants from aseptically germinated seeds of *Thymus piperella* L. were induced to form shoots on modified Murashige and Skoog medium, the best yield being 5.1 shoots per explant when the medium contained 6.6 M BA plus 2.8 M IAA. Shoots could be rooted on the same basal medium supplemented with 2.8 M IAA, and 71% of the plantlets were successfully acclimatized.

RESULTS AND DISCUSSION

Callus was induced in all media tested and no significant difference was observed in the induction frequency between light and dark conditions (Tables 1, 2 & 3). However, despite the similarity observed among the frequencies of callus formation, the differences in the callus type were noticed. Calluses induced under darkness and within the MS medium (Murashige and Skoog) containing 2mg/l 2, 4-D + 0.5mg/l Kn were friable and green in color. In all the treatments carried out in light conditions, the calluses were brown and compact mostly with dense areas, suggesting that the light treatment induce phenolic production in this specie. It is noteworthy, to mention that few species within the genus *Thymus* have been used to produce callus cultures. In these studies, the *T. sipyleus* (Bengi and Kamil, 1999) callus induction was obtained by cultivating seedlings in the presence of BAP + NAA. Callus induction and proliferation systems are known to be very useful for the study of biosynthesis of natural products and the factors that influence it, giving some possibilities of controlled production.

Table 1. Effect of auxin & cytokinin concentrations on callus initiation, shoot and root Proliferation of *Thymus vulgaris* L. from apical meristem explants cultured.

Growth Regulators (mg/l)	Max. height in(cm)	Max. number of shoots	Max. number of roots	Max. length of roots in (cm)	Number of days in response	Nature of response	In presence or absence of light
0.0 control	-	-	-	-	-	-	-
4 BAP	4	4	-	-	10	Shooting	*
2 2,4-D +0.5 Kn	6	5	-	-	32	Callus/shooting	*
4 BAP	14	10	-	-	7	shooting	#
1.1 BAP	4	3	2	8	30	Rooting/shooting	

Table 2. Effect of auxin & cytokinin concentrations on callus initiation, shoot and root proliferation of Thymus sipyleus L. from apical meristem explants cultured.

Growth Regulators (mg/l)	Max. height in(m)	Max. number of shoots	Max. number of roots	Max. length of roots in (cm)	Number of days in response	Nature of response	In presence or absence of light
0.0 control	-	-	-	-	-	-	-
5 NAA	9	1	-	-	14	shooting	*
0.4 NAA+ 3 BA	5	3	2	7	15	Rooting/shooting	*
0.3BA+3BAP	8	8	5	3	28	Rooting/shooting	*

Table 3. Effect of auxin & cytokinin concentrations on callus initiation, shoot and root Proliferation of Thymus piprella L. from apical meristem explants cultured.

Growth Regulators (mg/l)	Max. height in(m)	Max. number of shoots	Max. number of roots	Max. length of roots in (cm)	Number of days in response	Nature of response	In presence or absence of light
.0 control	-	-	-	-	-	-	-
6.6 BA+2.8 IAA	15	15	--	-	14	Shooting	*
2.8 IAA	8	4	7	5	25	Rooting/shooting	*
0.3 IBA + 3 BAP	8	8	5	3	28	Rooting/shooting	*

NAA	**Naphthalene acetic acid**	**BAP**		**Benzyl amine purine**	
IAA	**Indole acetic acid**	**(__)**		**No response**	
IBA	**Indole butyric acid**	**(*)**		**Presence of light**	
Kn	**kinetin**	**(#)**		**Absence of light**	
2,4-D	**2,4-dichlorophenoxyacetic acid**				

The results from the seeds germination studies indicated that herb can germinate in both the light and in dark conditions. However, the germination rate was higher in light as compared to darkness, so this necessity of light for germination depicts that the seeds are positively photoblastic. The germination and the percentage was enhanced to 100% by giving them moderate chilling treatment (up to 72 hrs) as against the treatment up to 45 hrs (80%) and up to 92 hrs (90%) (Table 4).

Table 4. Effect of different chemical and pre-chilling treatments on in-vitro seed germination

Seed treatment	No. of Seeds germinated		No. of days of germinated	(%) of response		Pre- chilling treatment in hours
	In light	In dark		In light	In dark	
0.0(control)	3/10	3/10	7	30	30	#
Thiourea	-	-	-	-	-	18
GA_3 200ppm	9/20	12/20	6	45	60	#
GA_3 +BAP 200ppm each	25/25	10/25	3	100	40	72
GA_3 +BAP 200ppm each	16/20	14/20	3	80	70	92

Control - With out treatment

GA3 - Gibberlic acid

BAP - Benzyl ammine purine

(-) - Non response

(#) - No pre- chilling treatment

This type of phenomena was also observed by Prasad (*1999*) on *Podophyllum hexandrum*. During the process of experiments, it was observed that seedlings raised showed contamination after 4 days of inoculation. The reason for this is that the seeds contain seed borne infection and when the seedlings grown from these seeds were transferred to MS medium containing nutrients, the infection grows easily on this nutrient rich medium. These experimental findings are clearly coinciding with previous studies. In these studies, it was observed that about 70% of the seedlings from the

heterogeneous seed were lost due to seed borne infection that grows easily on a nutrient rich medium. Similar results were observed by (Kiladas *et al.,* 1996).Mendes and Romano, (2004) also followed the same thing that the establishment of cultures was difficult due to high incidence of contamination for in vitro cloning of *Thymus-mastichina.*

Culturing the explants (apical meristem) on medium supplemented with 4mg/l BAP resulted in shoot induction (Table 1). BAP alone or in combination with NAA has previously been reported as being efficient in promoting shoot differentiation in several species (Blakesley and Constantive,1992). In *Thymus mastichina*, BAP was found to be the most efficient in shoot formation when nodal segments of mature field grown plants were used (Mendes and Romano, 2004). However, in case of T. sipyleus, the best results for shoot induction were obtained when excised parts of seedlings were cultured in media supplemented with BAP & NAA. In T. piperella, shoot and roots were induced in media containing BAP & IAA (Saez, *et al*., 1994).

The marked difference observed between the average numbers of shoots obtained from the explants induced either under dark or light conditions could be related to the media used for shoot induction and multiplication. Shoots induced under darkness resulted in significantly higher production of shoots than those induced under light (Table1). Shoots induced on 4mg/l BAP formed the highest number of shoots after 1 week of time. However, the shoots from the remaining treatments took 3 weeks to show shoot induction. The procedures that have established for shoot induction and multiplication of thymus species have reported an average from 4-7 shoots in 30-35 days, depending on the explants used. It is noteworthy, in mentioning that we reported a standard protocol which allows the production of up to 11 shoots per explant in 10-12 days.

It is well know fact, that rooting of shoots can be influenced by variations of medium salt strength and presence of growth regulators. Among the growth regulators, IBA, NAA, IAA has been the used to induce root formation in several species of thymus. In *T. piperelle*, the rooting was obtained in presence of IAA (Mendes and Romano, 2004). For *T. mastichina*, rooting was obtained in presence of NAA (Saez *et al*., 1994). In *Thymus vulgaris*, elongated shoots from explants were rooted in all media, although

higher frequencies were obtained in media supplemented with IBA + BAP (Table 2). The presence of IBA on the root media seemed to have effect only on the number of roots per shoot. Roots induced on the IBA containing media were thicker while those induced with out IBA were thinner. The regenerated plants were transferred to non-aseptic conditions for acclimatization and then to conditions of progressively lower humidity levels. Overall, it was observed that completely adapted plants were recovered after 20 days reaching 80% survival rate.

Fig.1

Fig.2

Fig.3

Fig.4

Figure 1-4d) Stages of micro propagation of *Thymus vulgaris*.

1. Shoot formation from apical meristem induced on 4mg/l BAP, under darkness
2. Shoots induced on root formation in MS medium supplemented with 0.3mg/l IBA + 3mg/l BAP
3. Callus formation from apical meristem cultivated on 2mg/l 2,4 2, 4-D + kinetin 2,4-D + 0.5mg/l Kn
4. and Seed germination by giving GA3 + BAP 200ppm each and pre-chilling at 72 hours.

CONCLUSIONS

The present study describes a new system which is suitable for micro-propagation and cell mass proliferation from apical meristem of *Thymus* sp. For multiplication of *Thymus vulgaris* selected clones, the apical meristem could be cultured under darkness on MS medium supplemented with 4mg/l BAP and shoots could be easily rooted on the MS medium containing 0.3mg/l IBA + 3mg/l BAP. For *Thymus sipyleus* Boiss sterilized seeds were germinated on Hellers medium, then these are transferred to MS medium

containing 0.4mg/l NAA & 3mg/l BA and then they formed callus when were in contact with the medium. For *Thymus piperella* germinated seeds were grown in MS medium containing 6.6Mμ BA + 2.8μM IAA. Shoots could be rooted on the same basal medium supplemented with 2.8μM IAA . 71% of plantlets were successfully acclimatized.

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