Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231– 6345 (Online)
An Open Access, Online International Journal Available at www.cibtech.org/sp.ed/jls/2015/01/jls.htm
2015 Vol.5 (S1), pp. 2903-2910/Golparvar et al.
Research Article

EFFECT OF PHENOLOGICAL STAGE ON YIELD, ESSENTIAL OIL AND THYMOL PERCENTAGE OF *THYMUS DAENENSIS* GROWN IN IRAN

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ABSTRACT
The genus *Thymus* L. belongs to the mint family (Lamiaceae), *Thymus daenensis*Celak is an endemic Iranian species. In order to optimum of harvest timethis plan was done in a randomized complete block design with three replications in research field ofIslamic Azad University, Isfahan (Khorasgan)branch, in center Iran, during spring and summer 2013. The aerial parts of *Thymus daenensis* were collected in five stages of plant growth, i.e. the before blooming, beginning of blooming, 50% blooming, full blooming and fruit set. The essential oil was extracted by a Clevenger approach and analyzed using GC/MS. Statistical analysis indicated that different stages of plant growth had significant effect on plant height, diameter, fresh and dry herbage, oil yield, thymol percentage and thymol yield of T. daenensis.Results of mean comparisons,fresh herbage revealed that the highest yield of fresh herb (10621.6 kg/ha) was obtained at the stage of fruit setand the highest yield of dryherb (4243.3 kg/ha) was obtained at the stage of fruit set. The highest oil percentage (1.41%) (v/w) was obtained at the stage of 50% blooming and lowest oil percentage (0.99%) (v/w) was obtained at the stage of before blooming. Analysis and identification of components showed thymol main compounds in all samples. The highest thymol content (84.1%) was obtained at the stage of before bloomingand the lowest thymol content (49.86%) was obtained at the stage of full blooming. The highest oil yield (46.7 kg/ha) and the highest thymol yield (31.62 kg/ha) obtained at the stage of fruit set.Results of this study suggest that at fruit set stage for T. daenensis optimum of harvest time on yield and quality of essential oil in Iran province.T. daenensis in semiarid condition is not rich in essential oil but rich in thymol, therefore farmers in semiarid regions should grow cultivated species for producing highest economic amount of extracted essential oil and thymol for pharmaceutical, therapeutic and food purposes.

Keywords: *Thymus Daenensis Celak, Harvest Time, Essential Oil, Thymolcontent*

INTRODUCTION
Thymus (thyme) is one of the most important genera with regard to the number of species withinthe family Lamiaceae. *Thymus daenensis* Celak,is an endemic species to Iran (Mozaffarian, 2008). The aerial parts and volatile constituents of thyme are commonly used as medicinal herb. Thymus species are commonly used as herbal tea, flavoring agents (condiments and spices) and for medicinal purposes (Stahl-Biskup, 2002). Infusion and decoction of aerial parts of Thymus species are used to produce tonic, carminative, digestive, antispasmodic, anti-inflammatory, rheumatism, skin disorders, expectorant and for the treatment of colds in Iranian traditional medicine (Zargari, 1989). GhasemiPirbalouti *et al.,* (2013) reported the main constituents of the oil (*ThumysdaenensisCelak*) were thymol (33.9–70.3%), carvacrol (4.0–24.8%), γ-terpinene (3.9–10.4%) and p-cymene (4.8–8.6%). For example, Golparvar *et al.,* (2014) reported effect of harvest time on quality and quantity of essential oil in *T. vulgaris,* the highest fresh weight (4964 kg/ha), the highest dry herbage weight (2564 kg/ha), the highest oil yield (43.4 kg/ha) and the highest thymol yield (24.24 kg/ha) were obtained at 50% blooming stage but the highest oil percentage (2.42%) was achieved in plants harvested at beginning of blooming stage. Studies reported by Safaei *et al.,* (2012) showed that in (*Thymus daenensis*Celak) the plants were harvested at four stages, the

highest aerial dry weight and oil yield occurred at full flowering stage and the highest oil percentage was recorded at 50% flowering.Studies reported by Omidbaigi *et al.*, (2010) indicated that more suitable time for harvesting of lemon thyme (*Thymus ×citriodorus*(Pers.) Schreb) to achieve the maximum yield of essential oil production is at fruit set stage.Sefidkon *et al.*, (2009) showed that it can be concluded that the beginning of flowering is the best for obtaining the higher oil content and thymol percentage thyme (*Thymus vulgaris* L.). McGimpsey *et al.*, (1994) have shown that seasonal variation has a significant effect on the yield and composition of thyme (*Thymus vulgaris* L.) essential oil. Jordan *et al.*, (2006) in a study about effects of harvesting times concluded also that in hymalian thyme (*Thymus hyemalis*Lange.) the highest percentage of thymol and carvacrol were obtained in beginning of blooming. Nejad-Ebrahimi *et al.*, (2008) find that the lowest essential oil percentage and the highest percentage of carvacrol were obtained in vegetative phase. Oil yield and phenol content peaked after flowering had finished. Harvesting time can be suggested according to area and its environmental conditions (Rey, 1991). Environmental differences among sites may cause differences in monoterpene production (Thompson *et al.*, 2003). Therefore, aim of the present work was to the effect of the time of harvesting (stage of plant development) of (*Thymus daenensis*Celak) grown in Iran on the yield of its herb, essential oil, and thymol.

MATERIALS AND METHODS

This experiment has been conducted in fieldIslamic Azad University, Isfahan (Khorasgan) branch, Isfahan, in center Iran (32˚, 38_ N and 51˚, 47_ E, 1550 m above sea level), during 2013 farming season on the base of factorial experiment in the layout randomized complete block design with three replications.

T. daenensis seeds were obtained from the Pakan Seed Company, Isfahan, Iran. The viability of seeds was approximately 90–95%. In the first week of January 2013, ten seeds were sown in glasshouse. The seedlings were transplanted to the filed Islamic Azad University, Isfahan (Khorasgan) branch on second week of March 2013. The soil of the field was clay loam with pH 7.37, contains total N (0.75 %), total P_2O_5 (35 ppm) and totalK_2O (452 ppm) with an EC of 4.69(ds/m).

The climate of regions in Isfahan (Khorasgan) is arid and warm area (according to the Koppen climate classification) characterized by warm and dry summers. The long-term (30 years) mean annual rainfall and temperature of area was 121.1 mm and 33.4 centigrade degrees, respectively. Transplant had been planted in rows 50 cm apart with inter-row spacing 20 cm.The samples were collected from annual plants in five stages, i.e. the before blooming, beginning of blooming, 50% blooming, full blooming and fruit set (15 July–20 September 2013).

Traits comprised height (cm), diameter (cm), fresh and dry herbage (kg/ha), oil percentage (oil weight obtained from 100 gr. dry matter) (v/w), oil yield (based on dry matter (kg/ha)), thymol percentage and thymol yield (thymol percentage oil yield (kg/ha)) were measured for every experimental unit.

Plants were cut at a height of 10 cm above soil levels and dried in a shaded area. Hundred gram powdered plant material was subjected to hydro-distillation (1000 ml distillated water) for 2 h using a Clevenger-type apparatus as recommended method in British pharmacopeiaSamples were dried using anhydrous sodium sulphate (Merck Co. Germany) and then kept in amber vials at 4 ± 1◦C prior to use.

GC/MS analyses were performed on a Thermoquest 2000 GC coupled with Thermofinnigan Mass system and a DB-1 capillary column (30 m_0.25 mm; 0.25 lm film thickness). The operating conditions were the same conditions as described above but the carrier gas was He. Mass spectra were taken at 70 eV. Mass range was from m/z 35–375 amu. Retention indices were calculated for all components using a homologous series of n-alkanes (C_5–C_{24}) injected in conditions equal to samples ones. Identification of oil components was accomplished based on comparison of their retention times with those of authentic standards and by comparison of their mass spectral fragmentation patterns (WILLEY/ChemStation data system) (Adams, 2007).

The data was statistically analyzed by $SPSS_{16}$ software, using completely randomized design (CRD). Means of the traits were compared by Duncan's multiple range test at $p < 0.05$ level. Analytical data for hierarchical cluster analysis were treated by means of the SPSS statistical software.

RESULTS AND DISCUSSION

Statistical analysis (Table 1) indicated that different stages of plant growth had significant effect on plant height, diameter, fresh and dry herbage, oil yield, thymol percentage and thymol yield of T. daenensis.

Table 1: Analysis of variance for different parameters

S.V	d.f	Plant height	Diameter	Fresh herbage	Dry herbage	Oil content	Oil yield	Thymol content	Thymol yield
Block	2	$6.49^{n.s}$	$16.28^{n.s}$	$135.94^{n.s}$	$2.54^{n.s}$	$0.000372^{n.s}$	$0.17^{n.s}$	$1.305^{n.s}$	0.326^{*}
Harvest time	4	19.47^{**}	1900.03^{**}	346.16^{**}	79.84^{**}	$0.493^{n.s}$	342.2^{**}	726.542^{**}	102.26^{**}
Error	8	8.01	83.63	132.03	61.34	0.000214	0.053	0.36	0.048

$^{n.s}$, *, **: non-significant, Significant at 0.05 and 0.01 probability levels, respectively

However, results of plant heightrevealed that highestplant height (31.83 cm) was obtained at the stage of fruit set and the lowest plant height (22.6 cm) was obtained at the stage of before blooming setting (Table 2). The highest diameter (38.5 cm) was obtained at the stage of fruit set and the lowest plant height (28.16 cm) was obtained at the stage of before blooming stage(Table 2).
The highest yield of fresh herb (10621.6 kg/ha) was obtained at the stage of fruit setting and the lowest (5545 kg/ha) was obtained at the stage of before blooming stage (Table.2). The highest yield of dryherb (4243.3 kg/ha) was obtained at the stage of fruit set and the lowest (2035 kg/ha) was obtained at the stage of before blooming stage (Table 2). Studies reported by Naghdibadi *et al.*, (2004) indicated that the maximum yield of dry and fresh herbage, yield and content of oil and thymol yield (*Thymus vulgaris* L.) were obtained in beginning of blooming stage, as well as Ozguven and Tansi (1998) showed that various harvesting times have significant effect on dry biomass weight and the highest dry matter was obtained stage of fruit set. The highest oil percentage (1.41%) (v/w) was obtained at the stage of 50% blooming setting and lowest oil percentage (0.99%) (v/w) was obtained at the stage of before blooming setting (Table 2). The highest oil yield (46.7 kg/ha) was obtained at the stage of fruit set and the lowest oil yield (20.26 kg/ha) was obtained at the stage of before blooming (Table 2).
Interaction effect of year and different harvesting stages in (*Thymus daenensis* Celak) showed that the highest aerial dry weight and essential oil yield (3083 and 68.61 kg/ha respectively) were recorded at full flowering stage in the second year. In both years, the highest amount of essential oil percentage was observed at 50% flowering (Safaie *et al.*, 2012).

Table 2: The effect of thyme harvesting time on different parameters in Iran province

Harvesting time	Plant height (cm)	Diameter	Fresh herbage (kg/ha)	Dry herbage (kg/ha)	Oil content (%)	Oil yield (kg/ha)	Thymol content (%)	Thymolyield (kg/ha)
before blooming	22.6_{b}	28.16_{d}	5545_{c}	2035_{b}	0.99_{a}	20.26_{b}	84.1_{a}	16.98_{b}
beginning of blooming	29_{ab}	31_{cd}	6541.6_{ab}	2235_{b}	1.25_{a}	28.22_{b}	73.96_{b}	20.8_{ab}
50% blooming	30.16_{a}	35.83_{ab}	7960_{abc}	3021.6_{ab}	1.41_{a}	44.82_{ab}	67.76_{c}	30.2_{a}
Full blooming	28.6_{b}	33.83_{bc}	9666.6_{ab}	3726.6_{ab}	1.19_{a}	44.56_{ab}	49.86_{d}	22.25_{ab}
fruit set	31.83_{a}	38.5_{a}	10621.6_{a}	4243.3_{a}	1.1_{a}	46.7_{a}	67.76_{c}	31.62_{a}

Means in each column followed by the same letter are not significantly different (P <0.05)

Qualitative and quantitative analysis of the essential oils volatile profile are listed in (Table 3). In T. Daenensis oil, 20, 22, 22, 25 and 23 compounds were identified in the before blooming, beginning of blooming, 50% blooming, full blooming and fruit set, respectively. The major constituents of the oil before blooming set were; thymol (84.1%), γ-terpinene (2.24%) and thymol methyl ether (1.32%). The major constituents of the oil beginning of blooming set were; thymol (73.96%), γ-terpinene (6.54%), carvacrol (4.77%), p-cymene (3.39%) and β-caryophyllene (2.28%). The major constituents of the oil 50% blooming set were; thymol (67.76%), carvacrol (6.76%), γ-terpinene (5.21%), α-terpinene (3.57%), β-caryophyllene (2.65%) and p-cymene (2.56%). The major constituents of the oil full blooming set were; thymol (49.86%), γ-terpinene (11.12%), carvacrol (9.15%), β-caryophyllene (5.87%),p-cymene (4.53%), linalool (3.72%), myrcene (3.18%) and borneol (2.54%). The major constituents of the oil fruit set were; thymol (67.76%), γ-terpinene (7.98%), p-cymene (5.31%), carvacrol (4.96%), and thymol methyl ether (2.01%).

Secondary compounds, including terpenes have multiple ecological functions. Many species in the genus Thymus show evidence of polymorphic variation in monoterpene production (Stahl-Biskup, 2002). Medicinal plants are no exception and their yield and composition of their essential oil are influenced by management practices such as harvest time as well as ecological and climatic conditions (Ozguven and Tansi, 1998). For example, Letchamo *et al.,* (1995) concluded that higher altitude and variation in soil type had profound effect in the spread and amount of volatile constituents of *Thymus serpylloides*. Ozguven and Tansi (1998) reported that the altitude should be considered as a major factor influencing the physiological and chemical characteristics of Thymus species.Analysis and identification of components showed thymol main compounds in all samples. The highest thymol content (84.1%) was obtained at the stage of before bloomingand the lowest thymol content (49.86%) was obtained at the stage of full blooming (Table 3). The highest thymol yield (31.62 kg/ha) was obtained at the stage of fruit set and the lowest thymol yield (16.98 kg/ha) was obtained at the stage of before blooming (Table 2). Highest oil yields and thymol yields resulted from plants at the stage of fruit set. This is due to high yields of fresh and dry biomass in this stage.

Result of Sefidkon and Rahimibidgoli (2002) about *Thymus kotschyanus* Boiss showed that full flowering was the best time to gain the highest essential oil amount. Nejad-Ebrahimi *et al.,* (2008) in their study on *Thymus caramanicus*Jalass observed that the lowest essence was obtained in vegetative phase (before flowering). They were reported that the yields of T. caramanicusoils (w/w) obtained from at different stages were 2.5, 2.1, 2.0, and 1.9% atflowering, floral budding, seed set and vegetative stages, respectively. Result of Jamali *et al.,* (2013) about (*Thymus maroccanus* Ball) in Moroccan showed that the yield of essential oils based on dry weight at different stages were; vegetative (2.14%), flowering (leaves: 1.80%; flowers: 3.46%), and post-flowering (leaves: 0.98%; post-flowers: 2.46%).

Essential oil biogenesis has also been linked to the levels of primary metabolites Unsuitable. Environmental conditions may limit photosynthesis in plants and alter nutrient uptake and carbon, sugar, amino acid and inorganic ion fluxes (Srivastava and Luthra, 1991). There are some minor differences in the relative amounts of other components in different satges of plant growth.

Safaei *et al.,* (2012) indicated that in T. daenensisof different harvesting stages in Isfahan, the highest amount of thymol was obtained from beginning of flowering stage (85.9%) and the highest amount carvacrol at fruit set. The maximum amount of P-cymene, 1,8-cineole and γ-terpinene were recorded at 50% flowering stage (3.4, 1.4 and 1.8% respectively). Studies carried out by Hudaib *et al.,* (2002) about evaluation of thyme (T. vulgaris) oil composition and variations during the vegetative cycle showed that the oil collected in May/June (phenological stage of FB–FR) was characterized by significantly lower levels of monoterpene hydrocarbons mainly α-terpinene and the highest levels of oxygenated monoterpenes linalool and borneol, monoterpene phenols (mainly thymol) and their derivates (mainly, carvacrol methyl ether), sesquiterpenes (mainly β - caryophyllene) and their oxygenated derivates (caryophyllene oxide) in comparison with all other samples.GhasemiPirbalouti et al., [3] reported the *T. daenensis* cultivated in Saman region produced highest thymol (70.3%), whereas those cultivated in

Shahrekord region produced highest oil yield (1.16%, v/w) and T. daenensis wild growing produced highest carvacrol (24.8%). The results indicated that essential oils and their chemical compositions of Thymus species are strongly affected by environmental conditions and agronomic management practices. Researches on garden thyme conducted by Naghdibadi *et al.*, (2004) showed that the highest thymol amount (47.99%) and thymol yield (55.28 kg/ha) were related to flowering initiation.

Table 3: The essential oil components of T. daenensis in different stage of plant growth

No	Compound	RI	Before blooming	Beginning of blooming	50% blooming	Full blooming	Fruit set
1	α- Thujene	932	1.23	0.86	1.76	0.32	0.42
2	α-Pinene	938	0.12	0.48	0.37	0.65	1.32
3	Camphene	948	0.47	0.59	0.15	0.71	0.14
4	Sabinene	974	0.95	0.13	0.01	1.38	0.35
5	β-Pinene	980	-	0.26	0.54	0.18	0.91
6	Myrcene	994	1.21	1.43	1.65	3.18	0.73
7	α-Phellandrene	1004	-	0.32	-	0.65	-
8	Δ-3-Carene	1010	0.05	-	-	-	-
9	α-Terpinene	1016	1.23	0.54	3.57	0.25	0.69
10	*P*-Cymene	1027	1.27	3.39	2.56	4.53	5.31
11	1,8-cineol	1031	0.89	0.14	0.84	0.38	0.25
12	γ-Terpinene	1065	2.24	6.54	5.21	11.12	7.98
13	(E)- Sabinene hydrate	1071	-	-	0.51	-	-
14	Terpinolene	1089	-	-	-	0.01	0.05
15	linalool	1098	0.89	0.54	1.46	3.72	1.43
16	Camphor	1143	0.07	0.06	-	1.15	-
17	Borneol	1167	0.71	1.32	0.32	2.54	1.12
18	Terpin-4-ol	1180	-	-	-	-	0.04
19	Thymol methyl ether	1235	1.32	0.56	1.87	1.67	2.01
20	Carvacrol methyl ether	1244	-	0.42	0.85	1.12	0.51
21	Thymol	1291	84.1	73.96	67.76	49.86	67.76
22	Carvacrol	1298	1.12	4.77	6.76	9.15	4.96

23	β-Bourbonene	1391	-	0.02	0.05	0.18	-
24	β-Caryophyllene	1414	0.78	2.28	2.65	5.87	1.91
25	Aromadendrene	1442	-	-	-	-	0.06
26	α-Humulene	1447	-	-	-	0.09	0.85
27	Germacrene-D	1478					
28	β-Bisabolene	1509	0.08	0.2	-	0.05	-
29	γ-Cadinene	1521	-	-	-	0.06	-
30	Δ-Cadinene	1529	-	-	0.09	-	-
31	E-α-Bisabolene	1545	0.36	0.08	0.03	-	-
32	Spathulenol	1577	-	-	-	-	0.01
33	Caryophyllene oxide	1580	0.54	-	0.09	0.87	0.56
	Total		99.63	98.89	98.59	99.69	99.37

RI = Retention indices in elution order from DB-5 column

The highest densities of alcohols, Ketons and steres were obtained in vegetative phase. Sefidkonet al., [19] observed that thymol amount was increased gradually in garden thyme from the first of vegetative phase to full flowering. Jordan *et al.*, (2006) in research of various harvesting times effects on essence quality and quantity of Himalayan thyme (*Thymus hyemalis*) observed that the highest density of gamma-terpinene (starter of *p*-cymene synthesis) was related to full flowering but the highest densities of thymol and carvacrol were obtained from full flowering and start of fruit ripening. Results of a study by Nejad-Ebrahimi *et al.*, (2008) indicated that the essential oils of T. caramanicus collected from Baft, Kerman province the highest content of carvacrol as major component (68.9%) was observed at full flowering stage, and the lowest content of carvacrol was observed at vegetative stage. They also were suggested at vegetative and seed set stages the amount of phenolic (carvacrol + thymol) portion was decreased, but the amount of their precursors (*p*-cymene + γ-terpinene) increased.Studies reported byMcGimpseyet al., [11] indicated that the highest oil and phenol contents resulted in plants harvested after full blooming stage. This variation may be referred to the seasonal variations that affected chemical constituents yields and contents in (*Thymus vulgaris* L.).Ghasemi Pirbalouti *et al.*, (2011) reported that the altitude showed be considered as a major factor influencing the chemical of (*Thumys daenensis* Celak) altitude seems affecting essential oil content of only oil rich and oil-intermediate aromatic plants. Changes regime of thymol percentage showed that this plant had highest thymol in vegetative phase but with passing vegetative phase and entrance to flowering phase, an obvious reduction in thymol amount occured, then in full flowering, thymol amount on the decrease that its reason could be various environmental and genetic factors. The results Semnani *et al.*, (2006) showed that the highest oil yield of *Thumyspubescens*obtained at full flowering stage.

The variability of thymol and carvacrolcontentsin the essential oils that obtained from *T. daenensis* cultivated and wild growing can be attributed mainly to environmental conditions as well as agronomic management including irrigation, plant density and soil tillage (Lubbe abd Verpoorte, 2011). Controlled growth systems also make it feasible to contemplate manipulation of phenotypic variation in the concentration of medicinally important compounds present at harvest (Canter *et al.*, 2005). Shortage of water in arid and semi-arid parts of this region where annual precipitation is less than 300 mm with almost no rainfall during the summer is a prominent limiting factor of Thymus production. Good management and adoption of suitable practices will improve economic Thymus production (Wang and Tian, 2004).

Conclusion

Results of this study suggest that at fruit set stage for T. daenensis optimum of harvest time on yield and quality of essential oil in Iran province.T. daenensis in semiarid condition is not rich in essential oil but rich in thymol, therefore farmers in semiarid regions should grow cultivated species for producing highest economic amount of extracted essential oil and thymol for pharmaceutical, therapeutic and food purposes.

ACKNOWLEDGEMENTS

This research project has been supported by Islamic Azad University, Isfahan (Khorasgan) branch, Isfahan, Iran. This support is highly appreciated.

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