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Review/Overview

Effect of plant spacing on herb, essential oil and artemisinin yields in the antimalarial herb (*Artemisia annua*)

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ABSTRACT

An experiment was conducted to work out the optimum spacing for obtaining maximum biomass, essential oil and artemisinin yields in antimalarial herb (*Artemisia annua* L.) in the semi-arid region of Gujarat. It was laid out in a randomized block design with 5 spacing treatments, viz. 30 cm × 45 cm, 30 cm × 60 cm, 45 cm × 45 cm, 45 cm × 60 cm and 60 cm × 60 cm. There were no significant differences in growth parameters like plant height and number of branches among different spacings. However, significant differences were observed in yield characteristics and was found that the mean fresh (30.79 t/ha) and dry (18.35 t/ha) herb yield and fresh (7.7 t/ha) and dry (3.91 t/ha) leaf yields, were maximum under spacing of 45cm × 45 cm. The leaf harvest index and leaf: stem ratio were also significantly higher under optimum spacing of 45 cm × 45 cm. No significant differences were observed in essential oil content, their composition and artemisinin contents among the different spacing but the yields of essential oil (54.95 kg/ha) and artemisinin (8.88 kg/ha) were found maximum in the spacing of 45 cm × 45 cm.

Key words : *Artemisia annua*, Artemisinin, Herb yield, Oil yield, Spacing

Artemisia annua (L.) (Family: Asteraceae), is an aromatic annual herb, traditionally grown in China as a medicinal plant. Artemisinin is the major compound present in *A. annua* and is used in the treatment of malaria. In Europe and India, *Artemisia annua* is used as a source of aroma chemicals for the fragrance industry (Sangwan *et al.*, 1998; Bakuni *et al.*, 2001) in additional to its medicinal value of artemisinin. The herb is also known to have antipyretic, antiparasitic, antiulcerogenic and anti-inflammatory properties (Sanja *et al.*, 2012). Since artemisinin is used for the treatment therapy against malaria worldwide, this compound is expensive and there is paucity of it in the world market (Enserink, 2005). The overall market size for artemisinin based treatments has been progressively expanded and by 2007 it has reached an estimated 150 million treatment courses, 20% of which is in the private sector. To meet the new artemisinin supply challenge, *A. annua* crop cultivation has to be expanded to different geographical locations. In India *A. annua* cultivation has been introduced and is being grown in the Indo Gangetic plains (Kumar and Srivastava, 2005). The cultivation practices also play a major role in the production of better herb, essential oil and artemisinin in *Artemisia annua* L. Plant density has been observed to have a huge influence on growth, development and yield of any crop. Different genotypes may respond variedly to different spacing. Essential oil content of *Artemisia annua* L decreases with increase in plant density (Tiwari, 2011). The higher plant population may compensate for reduction in seed yield (Kumari *et al.*, 2012). Since the plant spacing has a major role in production of a crop, we investigated to study the influence of planting density on herb, essential oil and artemisinin yields in *A. annua*.

MATERIALS AND METHODS

A field experiment was conducted at the Directorate of Medicinal and Aromatic Plants Research, Anand, (07°15'N and 78°14'E) Gujarat in two consecutive years (2008–09 and 2009–10. The soil of the experimental field was sandy loam with pH 7.8, electrical conductivity (EC) (dS/m) 0.24, available N 69.30 kg/ha, available P 41.51 kg/ha, available K 249.60 kg/ha and 0.31% of organic carbon.

The seeds were sown in the second week of December in both the experimental years in raised nursery beds and the cultural practices like mulching, irrigation and regular

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weeding were carried out. Germination was noticed 6 days after sowing and the seedlings were transplanted in the main field during the second week of February, 60 days after seed sowing, in both the years, at different spacing like 30 cm × 45 cm, 30 cm × 60 cm, 45 cm × 45 cm, 45 cm × 60 cm and 60 cm × 60 cm, with 4 replications under each treatment. The experiment was laid out in randomized block design, and the plot size for each replication in a treatment was 16 m^2. The beds were irrigated immediately after transplanting and further irrigations were given as required. No chemical fertilizers were applied. Only farmyard manure (FYM) @ 10 t/ha was applied as basal dressing in the experimental field. The growth and yield observations were recorded from the different replications of each treatment. The crop was harvested during full bloom stage in October in both the years (2009 and 2010) and the data on biomass yield, essential oil and artemisinin yields were recorded. The leaf : stem ratio was calculated as the sum of the dry weight of primary and secondary leaves originating from the stem sampled for each plot divided by the dry weight of the stem without leaves (Guan and Nutter, 2002). The leaf harvest index was calculated based on the formula leaf yield × 100/shoot mass (Kumar *et al.*, 2004). The data analysis was carried out using SPSS software.

For essential oil estimation the fresh sample (1 kg) in each replication was subjected to hydrodistillation using a Clevenger type apparatus for 4 hr. The distillate was extracted with diethyl ether, the ethereal layer was dried over anhydrous sodium sulphate and ether was removed on gently heated water bath and stored at 4–8°C until analysis. Analysis of the volatile oils was performed on a GC/MS (Focus-PolariQ) Benchtop Ion Trap Mass spectrometer equipped with a ZB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). Chromatographic conditions were as follows: injector temperature was 240°C, helium as carrier gas at a flow rate of 1 ml/min; injection volume was 1.0 µl (20 mg/ml in hexane). The column temperature was held at 60°C for 5 min., and programmed at 3°C/min. to 220°C and held for 5 min. with split mode injection (1:20) the MS transfer line and source temperatures were 240°C and 200°C. The GC column was coupled directly to PolarisQ quadrupole ion trap mass spectrometer in EI mode at 70eV with the mass range of 30–450 a.m.u at 1 scan/s. Kovat's retention indices were calculated using standard hydrocarbons (C_8-C_{20} n-alkanes, Sigma-Aldrich). The individual compounds (Table 3) were identified by mass spectra and their identities were confirmed by comparing their mass spectra with Mass Spectral Library (Ver. 2, 2005) and literature (Adams, 2007).

Artemisinin content in shade-dried and powdered aerial parts collected from each treatment were determined in duplicate. Aerial parts (1.0 g) was extracted with acetonitrile, filtered, evaporated using rotary evaporator and redissolved in 10 ml of acetonitrile. HPLC analysis was carried out by using Waters 600 E pump (Milford, MA, USA) attached to Waters 2996 photodiode array detector (PDA), Lichro CART® 250–4 C_{18} column (250´ 4.6 mm, 5 ìm; Merck, Germany), and on-line degasser. The data analysis was carried out using Empower software (Waters, USA). Acetonitrile and water (65 : 35) was used as mobile phase. Serial dilution of the 10µl of 1,000 ppm solution of each sample was injected with the flow rate of 1.3 ml/min. The amount of artemisinin was calculated by measuring the peak areas of 3 determinations using calibration curve of 50–2,000 ppm standard solution of artemisinin (>98%) purchased from Sigma Aldrich.

RESULTS AND DISCUSSION

There were no significant differences in plant height and number of branches among the different treatments (Table 1). The plant height ranged from 338 cm to 382 cm and number of branches ranged from 102 to 114/plant. However, earlier report showed that plant height in *A. annua* ranged from 50 cm to 300 cm (Woerdenberg *et al.*, 1994; Ferriera and Janick, 1996; Gupta *et al.*, 2002; Singh *et al.*, 2009). In the semi-arid region a vigorous plant growth was observed which may be due to the influence of climatic factors or soil properties.

The data on herb yield parameters like fresh and dry herb and leaf yield revealed that there were significant differences among the different spacing treatments in both the years (Table 1). The average fresh and dry herb yields was maximum under spacing 45 cm × 45 cm followed by 45 cm × 60 cm. Similarly, the average fresh and dry leaf yield (7.7 t/ha and 3.9 t/ha) was found to be maximum in the same spacing 45 cm × 45 cm. Our results contradict the findings of Ram *et al.* (1997) and Chauhan *et al.* (2008). In our experiment, the maximum herb and leaf yields were recorded in optimum spacing which allowed more plant spread when compared with minimum spacing 30 cm × 45 cm.

The leaf harvest index was the maximum under 45 cm × 45 cm spacing when compared with the other spacing treatments (Table 1). Leaf is the most economically important part of *A. annua* and the leaf mass is positively correlated with oil and artemisinin yield. In the spacing of 45 cm × 45 cm due to the optimum plant spread, the leaf production was more compared to the minimum spacing of 30 cm × 30 cm. Kumar *et al.,* (2004) reported similar result, who found that a negative correlation existed between leaf harvest index and shoot mass. The leaf: stem ratio was maximum (Table 1) under 45 cm × 45 cm spacing. In the optimum spacing of 45 cm × 45 cm, the plant

spread was also good, so that leaf production was more which correlated positively to the leaf stem ratio. In the lesser spacing 30 cm × 45 cm the plant height was good but the spread of the plant is affected due to minimum spacing which in turn resulted in minimum value for leaf stem ratio. Similarly, in higher spacing like 45 cm × 60 cm and 60 cm × 60 cm, though the plant spread was good but the number of plants per unit area resulted in lesser value for leaf: stem ratio. Kumar *et al*. (2004) also reported that leaves are the economic characters of *A. annua* and the proliferated plant growth did not favour economic yield in *A. annua* .

Similarly, no significant differences among the different spacing treatments on the essential oil content were observed and the yield of essential oil ranged from 0.65% to 0.7% (Table 3). In the present study, maximum essential oil content was found when compared to some of the previous reports (Ozguven *et al*., 2008; Bagchi *et al*., 2003). Besides the plant ecotypes differences, the influence of environmental, soil and climatic conditions may have affected the content and composition of secondary metabolites. From our study we found that the semi-arid region of Gujarat was highly suitable for growing *A. annua* for oil extraction, since the oil content and yield was more under these conditions. Though there were no significant differences in the oil content, significant differences were observed for the essential oil yield/ha in different treatments. Maximum oil yield (54.95 kg/ha) were recorded under 45 cm × 45 cm spacing (Table 2). Since the leaf yield and leaf harvest index were more in 45 cm × 45 cm spacing, it has resulted in higher essential oil yield compared to other spacing treatments. Mert *et al.* (2002) also reported that the ecotype Samakaya of *Artemisia annua* gave highest oil yield at spacing of 15 plants/ m^2.

Likewise, artemisinin content was also found non-significant in the different treatments and ranged between 0.39 and 0.42% (Table 2). The artemisinin content reported by us was comparatively lower than reported by Kumar *et al*., (2004). This may be due to influence of soil, climate, ecotype and environmental factors. However, significant variations in the artemisinin yield among the different spacing treatments were recorded. Highest artemisinin yield was recorded under 45 cm × 45 cm spacing, whereas minimum under 30 x 45 cm spacing (Table 2). The reason for higher artemisinin yield under 45 cm × 45 cm spacing is that when crops produce higher number of leaves with least stem growth, the leaf yield is high and ultimately results in higher artemisinin content (Kumar *et al.,* 2004). The leaf harvest index and leaf to stem ratio was maximum under 45 cm × 45 cm spacing, which resulted in higher oil and artemisinin yields in *A. Annua*, as they are concentrated in structures called trichomes, which

Table 1. Growth and yield parameters of *Artemisia annua* (pooled data of 2 years)

Treatment (Spacing)	Plant height (cm)	Number of branches	Fresh herb yield (t/ha)	Dry herb yield (t/ha)	Fresh leaf yield (t/ha)	Dry leaf yield (t/ha)	Leaf harvest index	Leaf: stem ratio
30 cm × 45 cm	360.4	105.5	23.8	13.4	5.3	2.1	18.8	0.20
30 cm × 60 cm	360.3	106.0	26.3	15.1	5.9	2.6	21.2	0.22
45 cm × 45 cm	354.5	106.8	30.8	18.4	7.7	3.9	25.8	0.31
45 cm × 60 cm	368.2	108.2	28.5	16.3	6.8	3.3	22.5	0.29
60 cm × 60 cm	356.1	108.4	26.2	15.0	6.2	2.9	20.5	0.25
SEm±	7.02	3.18	1.29	0.40	0.25	0.11	0.38	0.01
CD (P=0.05)	NS	NS	4.01	1.25	0.78	0.35	1.19	0.04

Table 2. Yield of essential oil and artemisinin content in *A. annua* (pooled data of 2 years)

Treatment (Spacing)	Essential oil (%)	Essential oil yield (kg/ha)	Artemisinin (%)	Artemisinin yield (kg/ ha)	Cost of cultivation (₹/ha)	Gross returns (₹/ha)	Net returns (₹/ha)	Benefit: cost ratio
30 cm × 45 cm	0.65	36.37	0.42	8.88	43,334	1,03,500	51,841	2.19
30 cm × 60 cm	0.67	42.48	0.39	10.21	40,650	1,21,500	77,251	2.90
45 cm × 45 cm	0.68	54.95	0.41	15.83	39,546	1,79,550	1,36,179	4.45
45 cm × 60 cm	0.67	48.17	0.39	12.80	38,285	1,48,950	1,08,641	3.84
60 cm × 60 cm	0.70	45.49	0.39	11.33	36,866	1,32,750	94,984	3.58
SEm±	0.02	1.97	0.01	0.58				
CD (P=0.05)	NS	6.15	N.S	1.80				

Dry leaf cost: ₹45/kg; Artemisinin: ₹40,000/kg; Essential oil: 900/kg)

Table 3. Percentage composition of the essential oil of *Artimesia annua* at different spacing treatments

Compound	RI	Area (%)				
		30 cm × 45 cm	30 cm × 60 cm	45 cm × 45 cm	45 cm × 60 cm	60 cm × 60 cm
á-Pinene	939	0.6	0.5	0.6	0.5	0.5
Camphene	954	0.8	0.9	0.8	0.8	0.8
Sabinene	977	1.3	1.2	1.3	1.3	1.3
b-Pinene	980	0.3	0.3	0.2	0.3	0.3
Myrcene	992	2.2	2.2	2.2	2.2	2.2
á-Terpinene	1,017	0.5	0.6	0.6	0.6	0.5
p-Cymene	1,026	1.1	1.1	1.0	1.1	1.1
Limonene	1,029	4.5	4.5	4.5	4.5	4.4
g-Terpinene	1,063	0.9	0.9	0.9	0.9	0.9
Terpinolene	1,082	0.2	0.2	0.1	0.2	0.2
1,8-Cineole	1,032	12.9	12.9	12.8	12.8	12.8
Camphor	1,149	28.6	28.7	28.9	28.6	28.6
Terpinen-4-ol	1,177	2.0	2.0	2.0	2.0	2.0
Myrtenol	1,197	0.2	0.2	0.2	0.2	0.2
trans-Carveol	1,218	0.3	0.2	0.2	0.3	0.2
Cumin aldehyde	1,238	0.2	0.2	0.2	0.2	0.2
Carvone	1,243	0.1	0.1	0.1	0.1	0.1
Thymol	1,291	0.1	0.1	0.1	0.1	0.1
p-Cymen-7-ol	1,292	0.1	0.1	0.1	0.1	0.1
Carvacrol	1,300	0.1	0.1	0.1	0.1	0.1
á-Copaene	1,377	0.3	0.3	0.3	0.3	0.3
b-Elemene	1,394	0.2	0.2	0.1	0.2	0.2
b-Caryophyllene	1,419	2.8	2.5	2.7	2.8	2.8
á-Humulene	1,455	0.1	0.1	0.1	0.1	0.1
trans-b-Farnesene	1,460	3.5	3.5	3.6	3.5	3.5
Germacrene-D	1,484	3.8	3.9	3.8	3.5	3.5
b-Selinene	1,490	0.4	0.4	0.3	0.4	0.5
Bicyclogermacrene	1,500	0.5	0.4	0.4	0.4	0.5
d-Cadinene	1,523	0.2	0.2	0.2	0.2	0.2
Spathulenol	1,580	0.2	0.2	0.2	0.2	0.2
Phytol	1,945	0.1	0.1	0.1	0.1	0.1
Eugenol	1,361	0.4	0.3	0.4	0.3	0.4
3-Hexen-1-ol	853	0.1	0.1	0.1	0.1	0.1
2-Methyl-butyl-2-methylbutanoate	1,103	1.1	1.1	1.0	1.0	1.0
Hexenyl-2-methylbutanoate	1,233	0.2	0.2	0.2	0.2	0.2
Phenylmethyl-3-methylbutanoate	1,390	0.2	0.2	0.2	0.2	0.2

RI, Retention index relatives to C_7-C_{32} *n*-alkanes on HP-1 capillary column;

is widely distributed in the leaves, inflorescence and shoot Ferreira *et al.*, (2005). The major compounds identified in GC-MS analysis under different spacing treatments is tabulated in Table 3. There were no differences observed in the distribution of the compounds in essential oil among the different spacing treatment combinations. Among the compounds identified, camphor (28.6–28.9%) and 1, 8–cineole (12.8–12.9%) using GC/MS were the major compounds present in the essential oil of *A. annua* (Table 3).

Economics

The highest net returns were recorded under 45 cm × 45 cm spacing, which again registered the significance of this plant spacing (Table 2). The net return and higher benefit: cost ratio were also observed under this spacing. The optimum spacing gives plants less competitive environment both above and below the soil that promotes better root growth and higher canopy development which finally results in ore leaf production and in turn more essential oil and artemisinin yield in *A. annua.* Similar kind of results were observed by Avasthe *et al.,* (2012) in rice, who observed higher net returns in optimum spacing of 20 cm × 20 cm in SRI cultivation system of rice.

The present study revealed that the optimum spacing of 45 cm × 45 cm is best suited for Gujarat condition for higher yield of herb, essential oil, artemisinin and in turn high net returns.

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