The effect of biological and chemical fertilizers on chlorophyll and artemisinin content in *Artemisia annua L.*

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Abstract

In order to consider impact of biological and chemical fertilizers (N, P) on chlorophyll and artemisinin content which exist in *Artemisia annua L.* an experiment was carried out in factorial design in completely randomized design with 4 replications in Zabol University in 2011. Treatments included chemical fertilizers (N, P) in 4 levels (N0P0, N40P40, N80P40, N80P80) and biological fertilizers in 4 levels (Control, Nitroxin [include bacteria which stimulus growth (*Azotobacter* and *Azospirillum*)], Bio-phosphorus [include bacteria which stimulus growth (*Bacillus* and *Pseudomonas*)] and Vermicompost fertilizer. Applying biological fertilizers lead to enhance active ingredient artemisinin in *Artemisia annua L.* and among biological fertilizers, Vermicompost has the most effect on enhancing artemisinin. The most increase in artemisinin content refers to Vermicompost + N80+P80 with (0.334%). Overall results of this experiment showed that the ability of *Azotobacter* and *Azospirillum* in stability N and capability of *Bacillus* and *Pseudomonas* in dissolution insoluble phosphates, enhanced artemisinin effectively. About chlorophyll concentration, applying biological fertilizers specially vermicompost has better impact that chemical fertilizers and Vermicompost + N80P80 had better impact on chlorophyll content in comparison with other experimental treatments.
Key words: Artemisinin, *Artemisia annua L.*, Nitroxin, Bio-phosphorus, Chlorophyll, Vermicompost

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Introduction

Artemisia annua (Asteraceae) is native to China, where it is known as qinghao (green herb) and has been used for over 2,000 years to treat symptoms associated with fever and malaria. It is known in the United States as sweet Annie, annual or sweet wormwood (Ferreira et al., 1997).

Malaria is a major health problem in many developing countries, mostly in Africa and Southeast Asia (Snow et al., 2005). According to WHO report on malaria (2007), 40% world's population is living with risk of malaria, over 1.5 million death occur per year and the cost of malaria treatment is $1800 million US dollar. The first effective ant malarial drug was quinine, which was isolated from the bark of cinchona. Since then malaria has been treated with quinoline based drugs. However, Plasmodium falciparum developed resistant globally against two of the most common ant malarial drugs: chloroquine and the combination sulphadoxine/pyrimethamine (Ridely, 2002).

Artemisinin, a sesquiterpene lactone containing a peroxide bridge, is isolated from the aerial parts of *Artemesia annua L.* plants and its derivatives are found effective against multidrug resistant *P. falciparum* strains (Krishna et al., 2004). In addition to potent anti-malarial

activity, artemisinin posses anti-cancer (Efferth et al., 2001), herbicidal (Chen and Zhang, 1987; Duke et al., 1987), anti-hepatitis B (Romero et al., 2005), anti-HIV (Jung and Schinazi, 1994), anti-leishmanial (Sen et al., 2007) and anti-schistosomiatic (Borrmann et al., 2001) activities.

Nitrogen (N) is an important element for growth of *Artemesia*. It needs N in large content which is a basic material for protein and nucleic acid. Studies which had been done by Ozguven et al (2008) and Singh (2000) showed that usage of different concentration of N has positive impacts on artemisinin content and its function. According to this fact that N participle in chlorophyll molecule structure directly, it could be expected that there exist a positive and significant relation between leave's N and chlorophyll content (Cassman et al., 1996).

Phosphorus (P) interferes with cells structure and most of vital activities such as storage and transfer chemical energy as well. Need for P in favor growth from 0.3 to 0.5% of dry weight is within growth and development stages (Ebrahim zadeh, 1994). Because N and P has been produced and used in chemical fertilizer form, its supply through using large content of chemical fertilizers in one of the water pollution in nature cycle and its production is expensive also, alternating this with organic fertilizers plays an important role (Chandrasekar et al., 2008). So that, avoid of negative pressure to environment, it is needed to improve developmental programs which supply plant fertilizers requirements'.

Improving soil quality could assesst according to quality and quantity index of biological society. As a result, using biological fertilizers is one of the effective managerial methods to keep soil quality in favorable level (Kokalis et al., 2006).

Using useful micro organism in agriculture had been begun since 60 years ago. Increasing this useful population can increase plant resistant against different environmental stresses such as lack of water, nutrition and heavy material toxicity (Wu et al., 2005).

Biological fertilizers are materials which include different micro creatures which have the ability to convert main nutrition elements from unavailable form to available form during biological processes (Rajendran and Devaraj, 2004) lead to develop better seeds' germination and root system (Bi et al., 2003).

In last decade biological fertilizers is applying as economically compatible compactly which lead reduction in using chemical fertilizers, improving soil fertility status to enhance plant production which is along with its biological activity in rhizosphere.

A group of bacteria which can be along with plant belong to Azospirillum, Azotobacter, Pseudomonas, Bacillus species (Selosse et al., 2004). Bacteria from Azotobacter and Azospirillum groups have the ability to make and leak some active and biological material such as vitamin B, Nicotinic acid, pentoterik acid, biotin, oxins, gebrelins etc in plant's root environment which have an effective and useful role in enhancement of root's absorbance (Kader, 2002).

Bacteria which work as solver of phosphate include a group of micro creatures most important species among this family is Pseudomonas and Bacillus (Tilak et al. 2005). Different species of Pseudomonas may cause to stimulate plant growth via different mechanisms such as antibiotics synthesis, plant hormone production, increasing P absorbance by plant, N stabling (Abdul-Jaleel et al. 2007).

Vermicompost is an organic biological fertilizer and consists of biological mixture of very active bacteria, enzymes, plant rests, animal fertilizer and soil worm capsule which cause continuation of soil organic material analysis and development of microbial activity in plant cultivation bed (Bashan and Holguin, 1997).

Artemisinin content in *Artemisia annua L*. is very low and its production in trade scale is not affordable, so universal attempts raise to increase its content. For example molecula,

physiological, breeding, biochemical and tissue culture techniques (Dong and Thuang, 2003; Ro et al., 2006; Zeng et al., 2007; Newmanet al., 2006; Zhang et al., 2009; Aquil et al., 2009; Weathers et al., 2005)
We also try to consider the effect of biological, chemical and their mixtures fertilizers on artemisinin and chlorophyll content in *Artemisia annua L*.

Methods and materials

In order to consider biological fertilizers (Nitroxin, Bio-phosphorus and Vermicompost) and chemical fertilizers (N, P) on chlorophyll and artemisnin content in *Artemisia annua L*., we had done an experiment in Zabol University green house in 2011. The plan of this experiment was factorial design in completely randomized design with 4 replications. Studding and considering chlorophyll content had been done in laboratory of biotechnology faculty of Jamia Hamdard University in India.

Experimented factors:

A. Biological fertilizers in 4 levels: A_1: controls (without using fertilizer), A_2: Nitroxin (include *Azotobacter* and *Azospirillum*), A_3: Biophosphorus (include *Bacillus* and *Pseudomonas*) and A_4: Vermicompost (10 t/ha). There existed 10^8 live cell in each gr of Nitroxin liquid and 10^7 cells in each gr of Bio-phosphorus liquid.
To mix and Insemination the seeds, firstly we extend clean plastic under seeds and then sprayed the liquid fertilizers on them. Then we put Inoculated seeds in shadow for 1 hour, after drying they are ready for cultivation, 10 tons Vermicompost also had been used.
B. Chemical fertilizer of N and P in 4 levels: B_1: Control (without fertilizer), B_2: N40+P40, B_3: N80+P40 and B_4: N80+P80 (Kg/ha).
Before cultivation, all the P fertilizers and N fertilizers in 3 parts added to pots according to soil test. Harvesting, samples did in April 2011 when 90% of plant have flowers.

The content of chlorophyll present in the samples tested

Weight 100 mg of leaf tissue in fractions into vial containing 7ml Dimethyl sulphoxide (DMSO). Chlorophyll will extract into the fluid without grinding at 65 C by incubating for various times, depending on the degree of customization and thickness of the leaf. Transfer the extract liquid to a graduated tube and made up to a total volume of 10 ml with Dimethyl sulphoxide (DMSO), assay immediately or transferred to vials and stored between 0-4 C until required for analysis. Take 3 ml of chlorophyll extract and transfer to curette,
Measure the optical density (OD) of the extract at the following wavelengths 645 and 663 nm using Dimethyl sulphoxide (DMSO) as a blank after 30 min, 1 hr, and incubation.
Calculate total chlorophyll as mg/g of tissue, using the following equations: [16]

Chlorophyll A (mg/g) =12.7 (OD663) – 2.69(OD645) x (V/ (1000 x wt))
Chlorophyll B (mg/g) =22.9 (OD645) – 4.68(OD663) x (V/ (1000 x wt))
Total Chlorophyll (mg/g) =20.2 (OD645) + 8.02(OD663) x (V/ (1000 x wt))
Where;
OD: optical density at certain wave length (645 or 663 nm)
V: final volume (10 ml)
Wt: weight of sample (100 mg)

Artemisinin extraction and estimation

Dry leaf material (1 g) was used for the estimation of artemisinin modified to a compound Q260 and quantified using HPLC method (Zhao and Zeng, 1986). Standard curve was prepared using 1 mg of standard artemisinin dissolved in 1 mL of HPLCgrade methanol to make the stock solution.

One g dry material was taken for extraction of artemisinin. It was extracted with 20 mL petroleum ether in shaker at 70 rpm for 24 h. After 24 h, solvent was decanted and pooled and 20 mL of petroleum ether added again and this step was repeated thrice. Petroleum ether fractions were pooled and concentrated under reduced pressure and residues defatted with CH3CN (10 mL×3). Precipitated fat was filtered out and filtrate concentrated under reduced pressure. Residues were dissolved in 1 mL of methanol. 100 µL aliquot of each sample of each treatment was taken and to this 4 mL of 0.3% NaOH was added. The samples were incubated in shaking water bath at 50°C for 30 min, thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% MeOH). The pH of the solution was maintained at 6.8. Derivatized artemisinin was analyzed and quantified through reverse phase column (C18; 5 µm; 4.6 mm; 250 mm) using premix methanol: 10 mM K-Phosphate buffer (pH, 6.5) in the ratio of 60:40 as mobile phase at constant flow rate of 1 mL/min, with the detector set at 260 nm. Artemisinin was quantified against the standard curve of artemisinin, obtained from Sigma–Aldrich, USA.

Statistical analysis

Statistical plan considered as factorial in completely accidental plot with 4 repetitions. Data analysis did by MSTAT-C and SAS software and graphs drew by excel software. In addition means compared in Duncan test and 0.05% probable level.

Results and discussion

Results of experimental data's statistical analysis are in table 1 and results of comparing considered characteristics means are in table 2.

Table 1: result of variance analysis of artemisinin, chlorophyll a, b and total in *Artemisia annua L.*

		(Mean Square)			
(S.O.V.)	**df**	**Artemisinin (%)**	**Chlorophyll a (mg/g)**	**Chlorophyll b (mg/g)**	**Total chlorophyll (mg/g)**
Repetition (R)	3	ns	ns	ns	ns
Bio-fertilizer (A)	3	**	**	**	**
Chemical fertilizer (B)	3	**	**	**	**
Bio-fertilizer × chemical fertilizer (A × B)	9	**	**	**	**
Error	48	0.000031	0.006	0.001	0.005
C.V. %		2.39	3.76	3.92	2.33

Note: *and ** indicate significant difference at 5% and 1% probability level, respectively
ns is not significant

Table 2. Comparison of experimental treatments' simple effects and interaction means on measured characteristics

Treatment	Artemisinin (µg/ml)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Bio-fertilizer (A)				
Control (A_1)	0.199d	1.89d	0.773d	2.66d
Nitroxin (A_2)	0.258b	2.235b	0.923b	3.15b
Bio-phosphorus (A_3)	0.225c	2.041c	0.807c	2.84c
Vermicompost (A_4)	0.278a	2.315a	1.06a	3.37a
Chemical fertilizer (B)				
Control (N0P0) (B_1)	0.183d	1.843d	0.761d	2.6d
N40P40 (B_2)	0.223c	2.006c	0.837c	2.84c
N80P40 (B_3)	0.266b	2.235b	0.955b	3.19b
N80P80 (B_4)	0.289a	2.4a	1.01a	3.41a
Bio-fertilizer × Chemical fertilizer (A ×B)				
Control (A_1B_1)	0.164n	1.773j	0.685m	2.45l
N40P40 (A_1B_2)	0.177m	1.847i	0.727l	2.57j
N80P40 (A_1B_3)	0.218i	1.948h	0.772k	2.72i
N80P80 (A_1B_4)	0.236h	2.011g	0.91f	2.92g
Nitroxin × N0+P0 (A_2B_1)	0.182l	1.79j	0.777k	2.56jk
Nitroxin × N40+P40 (A_2B_2)	0.245g	2.195e	0.881g	3.07f
Nitroxin × N80+P40 (A_2B_3)	0.291d	2.406d	1.004d	3.41d
Nitroxin × N80P80 (A_2B_4)	0.315b	2.549b	1.224b	3.773b
Bio-phosphorus × N0+P0 (A_3B_1)	0.174m	1.778j	0.747l	2.52k
Bio-phosphorus × N40+P40 (A_3B_2)	0.202k	1.882i	0.806j	2.68i
Bio-phosphorus × N80+P40 (A_3B_3)	0.254f	2.1f	0.822ij	2.92g
Bio-phosphorus × N80+P80 (A_3B_4)	0.270e	2.408d	0.853h	3.26e
Vermicompost × N0+P0 (A_4B_1)	0.211j	2.033g	0.836hi	2.87h
Vermicompost × N40+P40 (A_4B_2)	0.267e	2.104f	0.934e	3.037f
Vermicompost × N80+P40 (A_4B_3)	0.303c	2.489c	1.032c	3.521c
Vermicompost × N80+P80 (A_4B_4)	0.334a	2.634a	1.248a	3.881a

Note: Similar letters in each column hadn't any significant statistical difference.

The effect of chemical, biological fertilizers and interactions on artemisinin content

Results showed that chemical and biological fertilizers impact and their interaction on artemisinin which exists in *Artemisia annua L.* were significant in 1% probable level (table 1). According to means comparison (table 2) which shows biological and chemical fertilizers interaction an artemisinin, we can observe that by increasing N and P fertilizer along with biological fertilizers specially vermicompost, a significant ascending on this material appears (Figure 2).

The most enhancement of artemisinin (0.334%) is for applying chemical fertilizers (B_4) plus 10 tons vermicompost. After this treatment we can say the treatment which contains nitroxin (A_2) + chemical fertilizers (B4) locates in 2nd place with (0.315%) artemisinin.

The positive effect of vermicompost fertilizer on artemisinin content can explain as this fertilizer in addition to N contain K, P and other Micronutrients which all have positive effect on enhancement of artemisinin.

Comparison means of chemical fertilizers impact on artemisinin content shows that by increasing N and P, artemisinin will increase also (Figure 3). According to this result the most artemisinin content relates to (B_4) which are (0.289%).
About applying biological fertilizers, by considering means comparison (table 2) web find that, using biological fertilizers leads to artemisinin enhancement which among them, vermincompost (A_4) have better function cause, in increase artemisinin content to (0.278%) (Figure 4).
The significant increase in artemisinin content by the application of chemical fertilizers is also supported by Ozuven et al. (2008). They have reported significant increase in artemisinin content by the nitrogen application 120 and 80 kg ha^{-1}. In another study, Kapoor et al. (2007) reported that the application of phosphorus 30 kg ha^{-1} significatly increase the artemisinin content. Singh (2000) reported significant increase in artemisinin content by the application 50 and 100 N kg ha^{-1}. The positive effects of fertilizer application on secondary metabolites are in agreement with previous reports for other medicinal plants, such as, menthol mint (Ram et al. 2006), palmarosa (Rajeswara Rao, 2001), basil (Sifola and Barbieri, 2006) and fennel (Kapoor et al. 2004). One explanation for these observations is that nitrogen is a major constituent of several precursors (Ram et al. (2006). P content in plants also plays an important role in the biosynthesis of secondary metabolites (Liu and Zhong. 1998).

The effect of chemical, biological fertilizers and interactions on the Chlorophyll content (a, b and total) content

Results of variance analysis table indicate that using biological fertilizers has significant impact on chlorophyll in 1% statistic level (table 1). Results of means comparison in Duncan way showed that (table 2) among different level of biological fertilizers, the most chlorophyll content (a, b and total) relates to vermicompost (A_4) that are (2.315, 1.06 and 3.37 mg/g) and the less chlorophyll contents relates to control that are (1.89, 0.773 and 2.66 mg/g) (Figure 5).
Results show that the ability of biological fertilizers and using bacteria which stimulus growth are cause of enhancement chlorophyll in this treatment by fertility and by using bio-fertilizers we can increase chlorophyll content (a, b and total) and decrease chemical fertilizers usage significantly as well.
As you see in table 1, effect of chemical fertilizers on chlorophyll content was significant in 1% statistical level. Among different level of chemical fertilizer (table 2) the most contents of chlorophyll a, b and total were 2.4, 1.01 and 3.41 mg/g orderly, which related to B_4 that in comparison with control had 30.22, 37.12 and 26.69% enhancement (Figure 6).
In addition, interactions of biological and chemical fertilizers on chlorophyll content were significant in 1% statistical level (table 1). By comparison means comparison table (table 2) we observe that among treatments, the most chlorophyll content (a, b and total) relate to apply vermicompost (A_4) + N80+P80 (B_4) which were (2.634, 1.248 and 3.881 mg/g).
Nitrogen is closely related to the synthesis of chlorophyll (Salisbury and Ross 1992). Rubisco enzyme acts as a catalyst in CO3 fixation that plants need for photosynthesis (Salisbury and Ross 1992; Schaffer 1996). Therefore, total nitrogen content in plants can influence the outcome of photosynthesis via the photosynthetic enzymes and chlorophyll formation. In plants, nitrogen initially is in the form of ammonia and subsequent ammonia has been changed into glutamic acid, catalyzed by the enzyme glutamine synthetase (Harborne 1987). Glutamic acid serves as the base material in the biosynthesis of amino acids and nucleic acids (Nyakpa et al. 1988). Robinson (1980) called glutamic acid as a precursor for the porphyrin ring in formation of chlorophyll.

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