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PHYSIOCHEMICAL IMPACT OF A MAGNETIC FIELD ON *ARTERMISIA ANNUA* L. ALTERS ITS GENOMIC PROFILE AND ENHANCES ARTEMISININ CONTENT

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Abstract

Seeds of the Chinese herbal plant *Artemisia annua* L. were treated with a magnetic field (MF) prior to sowing in order to observe the effects on germination, height, growth, artemisinin content and change in genome profile. *A. annua* seeds were exposed to a magnetic field (MF) strength of 75 mT for different time intervals of 15, 30 and 45 min under laboratory conditions. The MF altered seed germination (87%), shoot length (63%), and artemisinin content (66%) in T45. The MF increased the seedling dry weight by 24.12 fold in T45. MF treated plants (T15, T30, and T45) also exhibited clear differences in PCR-RAPD profile; i.e. the genomic pattern as recorded in phylogenetic trees are 51%, 44%, and 44% for T45, T30 and T15, respectively. The most significant changes were observed in plants treated with a MF for 45 minutes, but all the recorded parameters were higher in treated seed raised plantlets. The increased germination, seedling growth and related chemical and molecular attributes could possibly enhance the artemisinin productivity of the plants. In future more research into the application of MF before sowing seed is needed to see if these results can be replicated in the field.

Key words: *Aretemisia annua* L., Artemisinin, Biomass, Magnetic field, PCR-RAPD.

Introduction

The use of magnetic fields (MF) has emerged as a novel tool in agricultural biotechnology to stimulate the biological clock of plants. This technique is far better than the use of chemical fertilizers and GMOs in terms of improved productivity and safety. It is an eco-friendly and non-polluting method for both the soil and the wider environment (Dhawi, 2014; Maffie, 2014; Iqbal *et al*., 2016). MF treatment of the seeds of various plants has previously been shown to affect plant growth, biomass, height and enzyme content. It is believed that it does not only to accelerate the nutrient uptake but also to affect the ability of plants to cope with environmental stresses by changing their biochemical reactions (Dhawi, 2014; Iqbal *et al*., 2016).

The derivatives of artemisinin have been used against many viruses, like human herpes simplex, and hepatitis B and C (Romero *et al*., 2005). Since cancer cells have a high iron content which is consonant with the activity of artemisinin, since iron and haem metabolism have a role in the anticancer activity of artemisinin (Zhang & Gerhard, 2009). Artemisinin derivatives have also been tested for their effect against human cancer cell lines (Romero *et al*., 2005). The derivatives of dihydroartemisinin, for example, have been proven to arrest the cell cycle and provoke apoptosis in specific cancer cell lines (Efferth, 2007). The growth of resistance to many longstanding drugs worldwide means that artemisinin is a particularly promising and important potential drug with applications across many diseases. The remarkably low yield of artemisinin is, therefore, a serious problem if it is to be used on a large scale globally (Abdin & Alam, 2015). Artemisinin and its derivatives are relatively costly due to the low quantity in the leaves of *Artemisia annua* L. plants. Many studies have been carried out by different investigators worldwide to enhance the level of artemisinin production either in plant systems or through chemical synthesis (Alam & Abdin, 2011; Alam *et al*., 2014). Although these efforts in plant tissue culture, breeding and genetic engineering have had a tremendous effect on artemisinin production (Wallaart *et al*., 2001; Delabays *et al*., 2002; Liu *et al*., 2003b; Graham *et al*., 2010; Paul *et al*., 2010; Alam & Abdin 2011; Townsend *et al*., 2013; Amanullah *et al*., 2016; Alam *et al*., 2016) but none of these efforts is feasible for the commercial production. We have therefore designed a method to overcome the challenges of artemisinin production at a mass level by effecting genome level changes to *A. annua* L. plants through the use of a magnetic field.

Materials and Methods

Plant material and electromagnetic field treatment: *Artemisia annua* L. seeds were gifted by Prof. M.Z. Abdin, Biotechnology Department, Jamia Hamdard, New Delhi, India. The seeds were germinated and maintained at a controlled temperature of 25ºC in plastic pots at Sara Alghonaim Research Chair, Biology Department, Prince Sattam bin Abdulaziz University, Alkharj, Saudi Arabia after treatment at a light intensity of 3000 lux in plastic pots containing peat moss, sand and vermiculite at a ratio of 1:1:1.

Exposure of *Artemisia annua* L. seeds to a magnetic field: A magnetic field (MF, Leybold Didactic GmbH, Huerth, Germany) was applied using coils with a tangential B-probe and high voltage and a current frequency of 220-240 V, 50/60 Hz. The strength was measured using a tesla meter. *A. annua* L. seeds were exposed to a MF prior to sowing following the method of Sharaf-Eldin *et al.,* (2015). *A. annua* seeds were divided into four groups, with each being loaded into 2 mL Eppendorf tubes placed within the ring of the electromagnet (Fig. 1). The *A. annua* L. seeds were then exposed to an MF of 75 mT for time intervals of 15 min (T15), 30 min (T30) and 45 min (T45). T0 was used as a control and was not exposed to the magnetic field.

Seed germination: Both the treated and non-treated *A. annua* L. seeds were germinated under controlled conditions at 25ºC in pots containing peat moss, sand and vermiculite at a ratio of 1:1:1. The seeds that germinated were calculated following Association of Official Seed Analysis rules (Carbonell *et al*., 2000). To estimate the germination percentage, plant seedlings visible at the substrate surface were recorded daily until further germination of the plant seeds ceased. The germination percentage was calculated.

Physiochemical parameters of MF treated *A. annua* L. plants: Physiological parameters like plant height, biomass (fresh and dry weight basis) were measured 45 days after the MF treatment.

Extraction and estimation of artemisinin: Extraction and estimation of artemisinin were carried out using the method of Khan *et al.,* (2015). In brief, dried leaves (a one gram sample) were extracted in 20 mL petroleum ether at 60ºC and kept for 24 hours at 28 ± 2°C shaking at 70 rpm. The supernatants were then filtered and the filtrate fractions were pooled and then evaporated to dryness using a rotary evaporator (Buchi, Switzerland). The residue was dissolved in 2.0 mL of methanol and kept at 4°C until HPLC analysis. Derivatised artemisinin was subject to HPLC (Water, USA) analyses using the reverse phase column (C18, 5 µm, 4.6*250 mm), and 100 mM K-Phosphate buffer (pH: 6.5) with a ratio of 60:40 used as a mobile phase, and with a 1 ml min^{-1} flow rate and a UV detector at 260 nm.

Extraction and purification of DNA: Leaf samples of MF treated (T15, T30 and T45) and non-treated *Artemisia annua* L. plants were processed for DNA isolation following the method of Doyle & Doyle (1990). The isolated DNA from T0, T15, T30 and T45 were quantified using a UV-VIS spectrophotometer (BMG LABTECH, Germany) at 260 and 280 nm adsorption. The quality of the extracted DNA was resolved on agarose gel (1% agarose) using a 1X TAE buffer, and the gel was visualized using the BioRad Gel Documentation system.

Generation of RAPD (Randomly amplified polymorphic regions) markers: Extracted DNA samples from the leaf samples of T0, T15, T30 and T45 of *A. annua* plants were subjected to PCR-RAPD analysis. PCR amplification was performed with ten sets of arbitrary GCC decamer primers (Table 1). Amplification reactions were carried out in a reaction volume of 25 µl containing the DNA template (15 ng/µl), dNTPs mix (2.5 mM each), 0.5 µl *Taq* DNA polymerase, reaction buffer (10X) and random primer (5 picomoles). The final volume was adjusted up to 25 µl with distilled water. The PCR amplification was performed in an Eppendorf thermal cycler, USA, using the following conditions: initial denaturation at 94ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 35ºC for 1 min and extension at 72º C for 2 min. Then the samples were subjected to a long extension at 72ºC for 5 min and, finally, a hold temperature of 4ºC. PCR products were resolved on 1.2% agarose gel and visualized using the BioRad Gel Documentation system.

Table 1. Arbitrary random primers used in RAPD analyses.

Name	Primers sequence
GCA01	CAGGCCCTTC
GCA02	TGCCGAGCTG
GCA03	AGTCAGCCAC
GCA04	AATCGGGCTG
GCA05	AGGGGTCTTG
GCA06	GGTCCCTGAC
GCA07	GAAACGGGTG
GCA08	GTGACGTAGG
GCA09	GGGTAACGCC
GCA10	GTGATCGCAG
GCA11	CAATCGCCGT
GCA12	TCGGCGATAG
GCA13	CAGCACCCAC
GCA14	TCTGTGCTGG
GCA15	TTCCGAACCC
GCA16	AGCCAGCGAA
GCA17	GACCGCTTGT
GCA18	AGGTGACCGT
GCA19	GTTGCGATCC
GCA20	CAAACGTCGG

Phylogenetic grouping using RAPD fingerprints: Ten decamer primer sets were used to amplify all possible polymorphic reproducible bands for initial screening against T0, T15, T30 and T45. *A. annua* MF treated plants were used to categorize PCR-RAPD markers. The phylogenetic relationships of the treated lines were developed based on Jacard's similarity coefficients (Jaccard, 1908) and UPGMA (unweighted pair group mean analyses). PyElph 1.3 software was used to construct the dendrogram.

Statistical analyses

All experiments were conducted on the basis of three replicates using one-way analysis of variance (ANOVA) followed by DMRT (Duncan's multiple range) applied using Graphpad prism ver. statistical software. The values are mean ± SE for three samples in each group. P-values at ≤0.01 were considered to be significant.

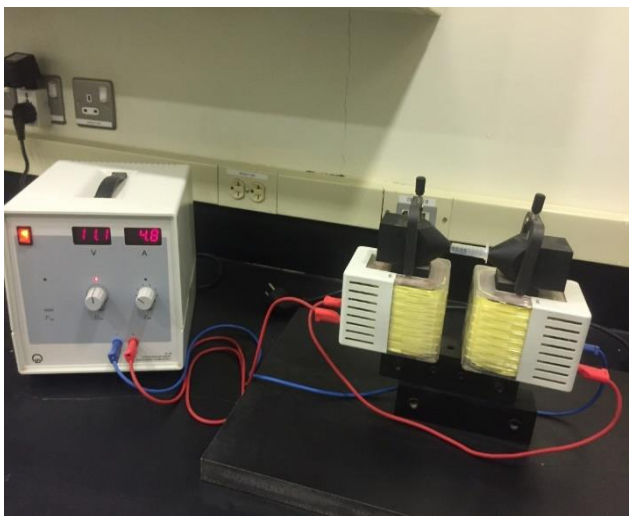

Fig. 1. Treatment of *A. annua* L. seeds under a magnetic field.

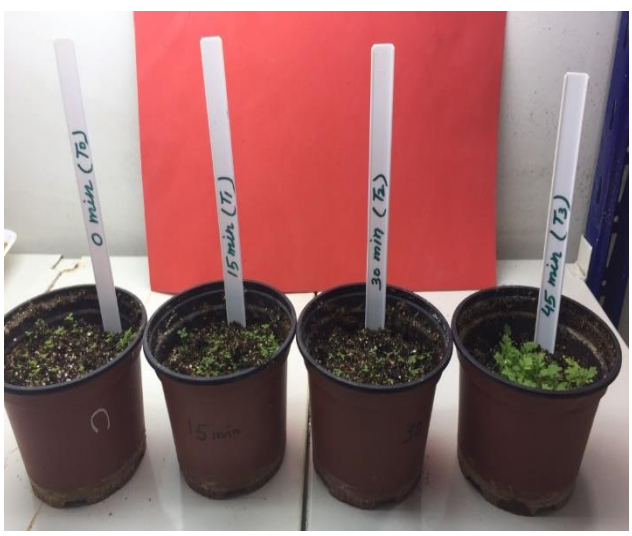

Fig. 2. Impact of MF on *A. annua* L. seed germination with different time intervals T15, T30 and T45 at 75 mT. T0 treated as a control.

Fig. 3a. Effect of magnetic field (MF) on plant height with different treatment time intervals T15, T30 and T45 at 75 mT. T0 treated as a control.

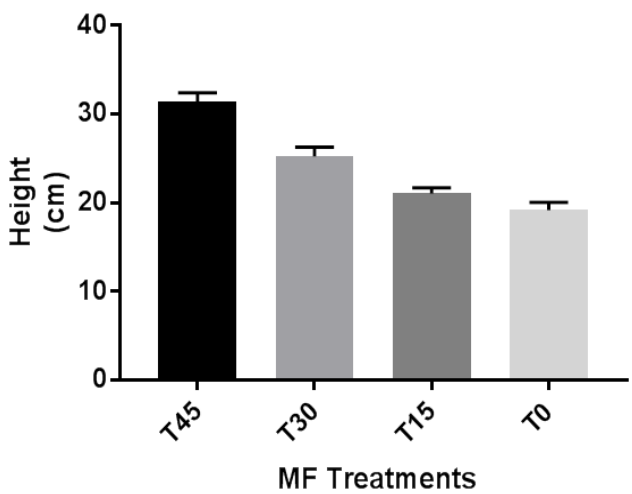

Fig. 3b. Effect of different time intervals of MF treatment on length (height) of *A. annua* L. T15, T30 and T45 at 75 mT. T0 treated as a control. Each value is the mean ± standard error (n = 3).

Results and Discussion

Physiochemical parameters: The interaction between living materials and electromagnetic radiation may (or not) induce an elevation of the tissue temperature, thus defining the thermal (versus non-thermal) associated metabolic responses. The best response of various growth parameters, seed germination, height, biomass and artemisinin content attributes were obtained using seeds of *A. annua* L. treated with MF prior to sowing. The *A. annua* L. seeds were treated with three time intervals (15, 30 and 45 min) and the percentage rate of germination was recorded. The maximum germination percentage of all MF treated seeds (100 seeds) was recorded after germination of the seeds. After the tenth day following germination in pots containing sand, peat moss and vermiculite (1:1:1), the germination percentage was recorded as 87% in the T45 treatment group, 75% in the T30 group, 68% in the T15 group, compared to 60% in the untreated (T0) group (Fig. 2).

Germination and emergence of seedlings for respiration places a high demand for energy on the seed embryo and cotyledons. The stimulation of seed germination might be attributed to a combined effect of biochemical, physiological, free radical mobile electron charges, metabolic processes and enhanced enzymatic action. It is considered that a MF can alter cell membrane permeability and transfer water and energy signals into the cell (Reina *et al.*, 2001) thereby influencing metabolic pathways (Podleoeny *et al.*, 2004; Iqbal *et al.*, 2013). The enzymes which are necessary for particular stages of seed germination have also been found to be higher in magnetically treated seeds (Vashisth & Nagarajan, 2010).

The growth of all seedlings was recorded after the tenth day of transplanting into plastic pots containing the same mixture of soil as for germination with controlled conditions at 25ºC. The growth of all the *A. annua* L. seedlings produced from treated seeds was significantly enhanced compared to those produced from non-treated seeds. For instance, the seedling height of T45 seeds was 31.43 cm in magnetized seeds compared with 25.26 cm for T30, 21.06 cm for T15 and 19.2 cm for the control T0 (Fig. 3a, 3b).

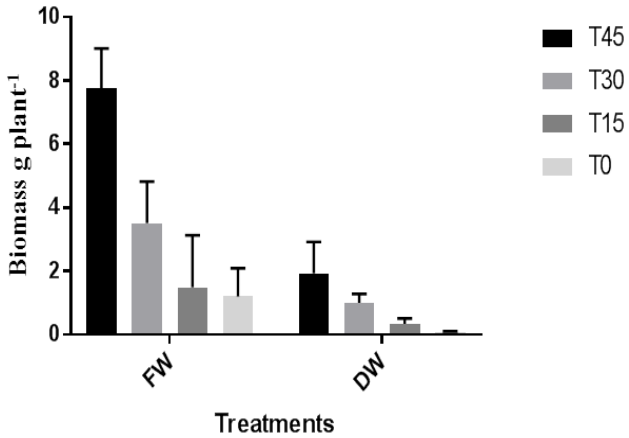

Fig. 4. Effect of MF treatment on biomass (g plant-1) of *A. annua* L. according to different time interval T15, T30 and T45 at 75 mT. T0 treated as a control. Each value is the mean ± standard error (n = 3).

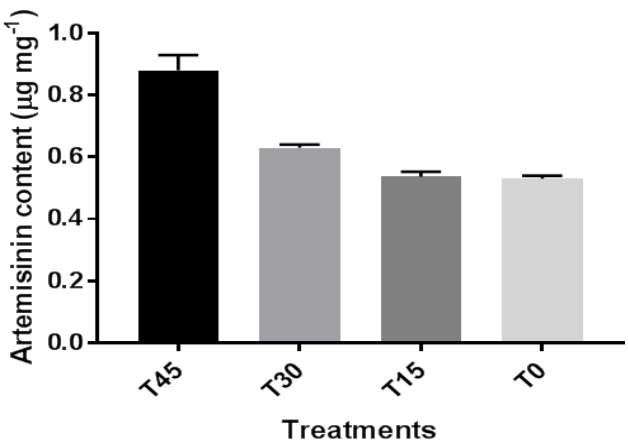

Fig. 5. Effect of different intervals of MF treatment (T15, T30 and T45 at 75 mT) on the artemisinin content of *A. annua* L. T0 treated as a control. Each value is the mean ± standard error (n = 3).

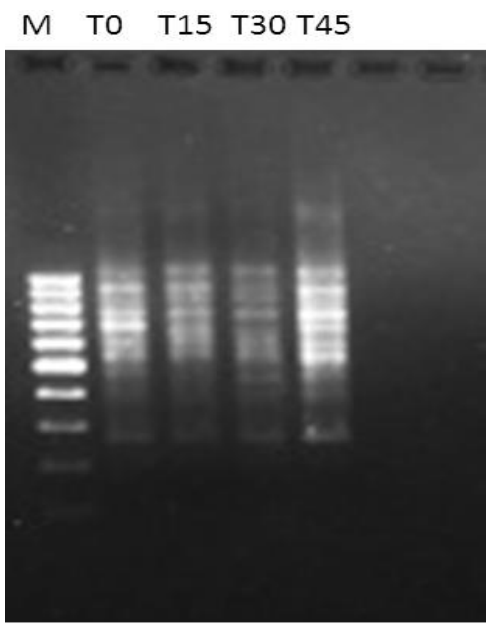

Fig. 6. PCR-RAPD profile of MF treated plants of *A. annua* L., M: 100 bp DNA ladder with different time intervals T15, T30 and T45 at 75 mT. T0 treated as a control.

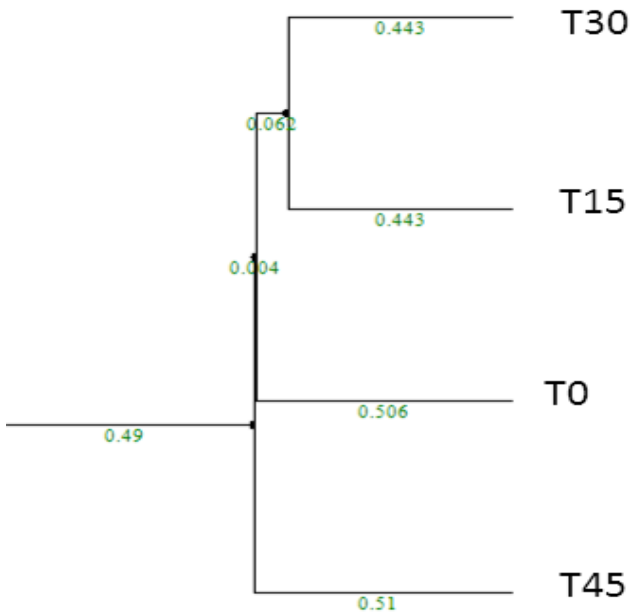

Fig. 7. Phylogenetic tree of *A. annua* L. plants treated with a MF of 75 mT at different time intervals of T15, T30 and T45. T0 treated as a control.

Significant differences ($p \leq 0.01$) in the biomass accumulation per plant were observed on both a fresh and dry weight basis between MF treated and non-treated plants. A maximum fresh biomass of 7.75 g was recorded in plants subject to MF treatment for 45 minutes (T45), compared with 3.51 g for T30, 1.48 g for T15 and 1.2 g for T0. The same pattern was recorded for the dry biomass: 1.93 g for T45, compared to 0.08 g for T0 followed by T15 (0.33 g) and T30 (0.99 g) (Fig. 4). Thus the MF increased the seedling dry weight by 24.12 fold.

The progressive effect of MF treatment in terms of increasing the rate of germination and the height and weight of shoots was also observed in maize by Aladjadjiyan (2002). Similar experiments were also carried out by Boe & Salunkhe (1963) and De Souza *et al.*, (2006). They observed that MF acts like a plant hormone and proposed that it served to activate or accelerate enzymes related to auxin reactions in plant system leading to increased growth (Taia *et al*., 2007; Maffie, 2014).

The artemisinin produced by treated and non-treated *A. annua* L. plants was determined 45 days after planting using HPLC (Waters, USA) as described by Khan *et al.*, (2015). The area of the relevant peak of each sample was interpolated with the standard curve to determine the quantity of artemisinin in the leaf (µmg-^{1} dry wt). The artemisinin peak was detected at 14 min (RT) using a phosphate buffer (100 mM): MeOH (4:6) as the solvent system and a 125 x 4 mm C18 column. The artemisinin content was 0.88 µmg-^{1} dry wt in T45 treated plants and 0.53 µmg-^{1} dry wt in T0 (control) plants. Consequently, there was a 66% increment in the artemisinin content of plants treated with MF for 45 minutes compared to the control (Fig. 5). MFs are known to play an important role in cell growth, development and multiplication (Yokatani *et al*., 2001). The MF affects charged particles in a phenomenon called geotaxis or electrotropism (Wagner, 1999). The geotaxis induced by an MF attracted the

charged particles and the free radicles within plant cells, which influences the plant growth and accumulation of ions, as well as leading to increased photosynthetic pigments and stress reaction represented in proline biosynthesis which accelerate *A. annua* L. plants growth. Several studies other studies have reported similar results in other plants (Yokatani *et al.*, 2001, Celestino *et al*., 2000, Nasher, 2008).

Molecular differences: gDNA was isolated from each treatment (T45, T30, T15) and control group (T0) of *A. annua* L. using the protocol of Doyle & Doyle (1990). The DNA was made free from contaminants such as RNA, proteins, phenols, terpenes and other secondary metabolites, as well as the phenolics present in the leaves of *A. annua* L. plants. The quality and quantity of the isolated DNA were determined using a Bichrome (UK) spectrophotometer at 260/280 nm. The results revealed that the yield of DNA varied between 171 ng and 150 ng in *A. annua* L. treated and control plants, respectively.

The purified DNA was used for PCR-RAPD in order to analyse the effect of the MF at the genome level. Ten random GCC primers were used for the RAPD analysis. The RAPD profile showed a monomorphic (similar band), and a polymorphic (unique) band with the selected random GCC primer. The size of the bands varied by more than 1000 bp in both the monomorphic and polymorphic bands (Fig. 6). The amplification pattern was found to be more pronounced with the GCA-02 (TGCCGAGCTG), while the remaining random primers were unable to amplify the gDNA of *A. annua* L. plants uniformly.

On the basis of the polymorphism between the treated and non-treated *A. annua* L. plants, a similarity matrix was obtained after multivariate analysis using the "Nei and Li" coefficient (Nei & Li, 1979). The results of the genetic similarity matrix coefficient indicate that *A. annua* L. had about 51%, 44%, and 44% (T45, T30 and T15) similarity with all treatment and control, respectively.

The dendrogram of *A. annua* L. constructed on the basis of this PCR-RAPD clearly indicates that the similarity and distance between the treated and untreated *A. annua* L. through which one could visualize the source of the species more clearly (Fig. 7), and revealing the mutational pattern related to the treatment.

The negative charges of DNA molecule potential of MF treated seeds of *A. annua* L. affected the molecular level of plant (Atak *et al*., 2003; Dhawi & Al-Khayri, 2009). MF induced changes at the cellular level have been shown to lead to an increase in cell viability, organization and differentiation (Sahebjamei *et al*., 2007). In addition, MF affects cell reproduction and cellular metabolism, gene expression and enzyme activity (Phirke *et al*., 1996; Paul *et al*., 2006). These findings were comparable with earlier studies showing that MF influences plant growth and development at lateral stages of *A. annua* L. plants.

MF doses of 75 mT revealed early growth in *A. annua* L. plants, significantly ($p \leq 0.01$) higher shoot length, biomass and artemisinin content. This is due to changes at the genome level with over expression of some genes in the plant (Carbonell *et al*., 2000; Flórez *et al.*, 2007; Vashisth & Nagarajan, 2010; Iqbal *et al.*, 2013; Shabrangi *et al.*, 2013). Similarly, De Souza *et al.*, (2014) recorded higher plant height in onions as a result of extremely low frequency non-uniform magnetic field treatment, and broad bean seeds treated with MF showed early seedling growth in comparison to the control (Rajendra *et al*., 2005).

Overall, therefore, the results of this study suggest that MF may enhance the artemisinin content in treated plants.

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