Available online: www.notulaebotanicae.ro
Print ISSN 0255-965X; Electronic 1842-4309
Not Bot Horti Agrobo, 2016, 44(1):41-47. DOI:10.15835/nbha44110328
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
AcademicPres

Original Article

High Frequency Pulsatile Electromagnetic Fields and Ultrasound Pulsatile Fields Impact on Germination Dynamic of *Ocimum basilicum* L. and *O. basilicum* var. *purpurascens* Benth., Observed with Open Source Software

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Abstract

Basil (*Ocimum* sp.) is generally known as "king of herbs" due to its pharmaceutical and culinary properties. Even though *Ocimum* sp. are considered to have fast germination velocity index (GVI), short mean germination time (MGT) and increased seedling vigor index (SVI) values, their experimental monitoring is usually done with specialized software's that are expensive. Low cost scientific solutions are keen on open source software germination protocols. For observing the accuracy of open source, C.A.D. softwares were used for *Ocimum* sp. seeds which were submitted to high frequency pulsatile electromagnetic fields (300 impulses/3 peak penetrance - 293 W) and ultrasound pulsatile fields (1/2 pulses by 0.5 W/cm^2). All sequence images where taken using a positive/negative photo filter enhancing the differences in seed development. At the end of the experiment, all images where transformed into vector formats (dwg). On the dwg extension, selective free plug-ins such as Face Centroid and Area Properties helped to collect data like seed development on X/Y scale, area, perimeter, no. of germinated seeds, length of seedling root, hypocotyl length. Mean germination time (MGT) was in agreement with the accepted theory of seed germination that enounced a diametric position between values of GVI and MGT, meaning that all the experimental variants with high values of GVI recorded low values of MGT. All the experimental variants influenced by physical stimulants recorded improved contents of therapeutical terpenes.

Keywords: monitoring, development, digital, raster, vector

Abbreviations: GVI - germination velocity index, MGT - mean germination time, SVI - seedling vigor index

Introduction

The *Ocimum* genus belonging to Lamiaceae family has a widely spread genetic diversity with at least 65 botanical classified species (Paton *et al.,* 1999). Other studies enlarged the botanical classification within the range of 150 species (Sahoo *et al.,* 1997). The main origin of these species derived from the tropical and sub-tropical regions of Asia, Africa, Central and South America with a main center of diversity in Africa. The botanical name significance of *Ocimum* genus has its base point in Greek language meaning "to be fragrant". From this point of view, the main usage of basil consists in food industry and pharmaceutical sectors, due to its content of monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins, and phenolic acids. In addition, extracts of *Ocimum* genus are commonly used in traditional medicine for insecticidal, nematicidal, fungistatic and antimicrobial properties (Zlotek *et al.,* 2016). Taxonomy issues such as numerous botanical varieties, cultivar names and chemotypes make *Ocimum* genus hard to classify (Simon *et al.,* 1990).

Although vegetative and micro-propagation methods represent key solution for uniform and constant yields, in order to understand the diversity and taxonomy of a gender, seed propagation always seems to be on first approach. For technological reasons, seed propagation is keen on minimum inputs solutions, assuring well-balanced cost-profit ratios. In order to observe the influence of physical treatments upon seeds' germination, *Ocimum basilicum* L. and *O. basilicum* var. *purpurascens* Benth. seeds where exposed to high frequency pulsatile electromagnetic fields (300 impulses/3 peak penetrance - 293 W) and ultrasound pulsatile fields (1/2 pulses by 0.5 W/cm^2). The main scientific hypothesis of this experiment consists in observing the germination behavior of seeds subjected to physical stimulation, and if their reaction to these stimuli is to lead to improvement of monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins and phenolic acids content in mature plants. Even though the influence of physical stimulation upon seeds is generally unknown, several theories have prevailed. According to Vashisth and Nagarajan (2008), Pietruszewski and Kania (2010), some physiology seed aspects regarding intra- and extracellular ionic movements, assure the restoration of

Received: 17 Mar 2016. Received in revised form: 10 June 2016. Accepted: 12 June 2016. Published online: 14 June 2016.

bioelectric potentials in the cell membranes, thus activating seed germination. Other hypotheses are keen on water dipole movements under the presence of magnetic fields (Moon and Chung, 2000; Matwijczuk *et al.,* 2012; Ahamed *et al.,* 2013;; Occhipinti *et al.,* 2014). The same movements are general accepted for chemical nutrient intakes. According to Dhawi *et al.* (2009) and Esitken *et al.* (2004) magnetic fields presence plays an important role in cation uptake capacity. Cation uptake capacity is explained in several studies as changes to root gravitropism (Kordyum *et al.,* 2005). Magnetic field influence observed at detailed level with the usage of a digital microscope, increased cellular division at meristematic level (Belyavskaya *et al.,* 1992). Magnetized water helped wheat cultivars to resist salt stress conditions, assuring a better salt tolerance than control experimental variants (Al-Mashhadani *et al.,* 2016).

Regarding seed development, MGT (mean germination time) was considerably reduced when static magnetic field was applied to *Salvia officinalis* L. and *Calendula officinalis* L. seeds (Flórez *et al.,* 2012). Same results with reduced MGT values where obtained for *Pinus taeda* L. at 150 mT for 60 minutes (Yao and Shen, 2015). In different experimental plots, in order to observe the seed morphological movements under the influence of magnetic fields, seeds where glued with non-toxic adhesive to filter paper with their long axes vertical. By this method, Florez *et al.* (2007) observed in dynamic morphological aspects of seed development.

Many magnetic experimental plots for seed germination, relate that an optimum amount of magnetic energy and exposure is applied to different gender seeds. For example, at *Urtica dioica* L., an optimum intensity and duration was set on 0.8 mT for 20 minutes (Rostami *et al.,* 2014).

The second physical treatment for accelerating seed germination in the proposed experimental plot consisted in ultrasound pulsatile fields, generally known as "sonication" (Shekari *et al.,* 2015). When ultrasounds are applied to seeds, the scientific process of germination is called "ultrasonication" (Liu *et al.,* 2016). The ultrasound pulsatile fields are presented in the scientific literature as methods of breaking seed dormancy of thick tegument seeds. Synergic effects consider ultrasound pulsatile fields decontaminating agents (Chiu and Sung, 2014; Jeong *et al.,* 2014).

Like in the case of electromagnetic pulsatile fields, seed germination in ultrasound pulsatile fields depends on the frequency, intensity and duration of sound irradiation (Goussous *et al.,* 2010; Toth *et al.,* 2012; Wang *et al.,* 2012).

The main goals of the present study were keen on observing the following aspects: (1) effect of high frequency pulsatile electromagnetic fields upon seed germination of *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth.; (2) effect of ultrasound pulsatile fields upon seed germination of *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth.; (3) digital observation of seed development; (4) the effect of high frequency pulsatile electromagnetic fields and ultrasound pulsatile fields upon main terpenes quantity.

Materials and Methods

Biological material

The biological material represented by *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. seeds, was granted by a BGCI (Botanic Gardens Conservation International) exchange between Botanical Garden of USAMV Cluj-Napoca, Romania and Orto Botanico dell'Università di Siena, Italia.

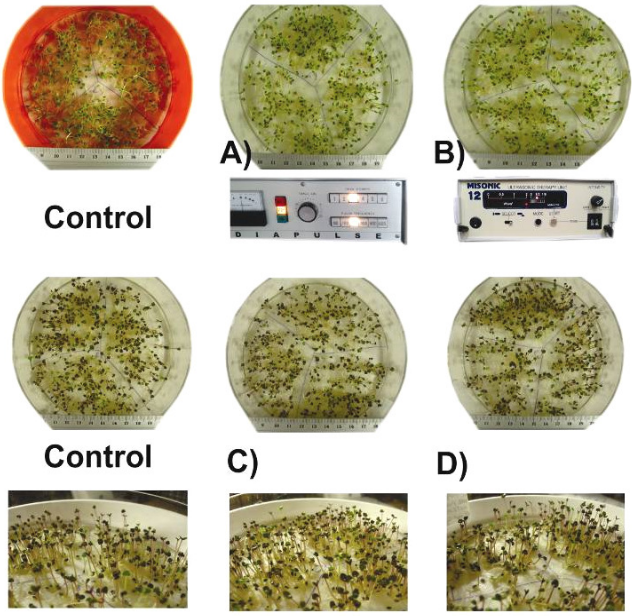

Fig. 1. Experimental plot design seed germinators; A) *O. basilicum* L. high frequency pulsatile electromagnetic fields; B) *O. basilicum* L. ultrasound pulsatile fields; C) *O. basilicum* var. *purpurascens* Benth. high frequency pulsatile electromagnetic fields; D) *O. basilicum* var. *purpurascens* Benth. ultrasound pulsatile fields (original)

Experimental design

A completely randomized experimental design with four replications was used. Germination was recorded daily, the evaluation criteria being the presence of a radicle of at least 2 mm. Four replicates of 100 seeds for each treatment where used. Germination tests were performed according to the International Seed Testing Association (ISTA, 2014).

The experimental factors where: (1) biological material – *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. (Fig. 1); (2) germination method - distilled water (control), high frequency pulsatile electromagnetic fields (300 impulses/3 peak penetrance - 293 W); ultrasound pulsatile fields (1/2 pulses by 0.5 W/cm^2).

The experimental plot was emplaced in the laboratory of Computer Assisted Design Department, USAMV Cluj-Napoca, Romania. Seed germination tests were conducted in standard Linhard germinators lined with distilled water moistened cellulose wadding. During the experiment period, water level in germinator reservoir was maintained by inputs of distilled water. In order to maintain optimum germination conditions microclimatic factors where evaluated daily with a digital multi-meter, assuring constant values for all the experimental factors: relative air humidity (60%), germination temperature (25 °C), light intensity (195 lux).

After germination tests, the resulting seedlings where transferred in vegetation pots at the green house of Ornamental Plants Department, USAMV Cluj-Napoca, Romania, where they received standard vegetation

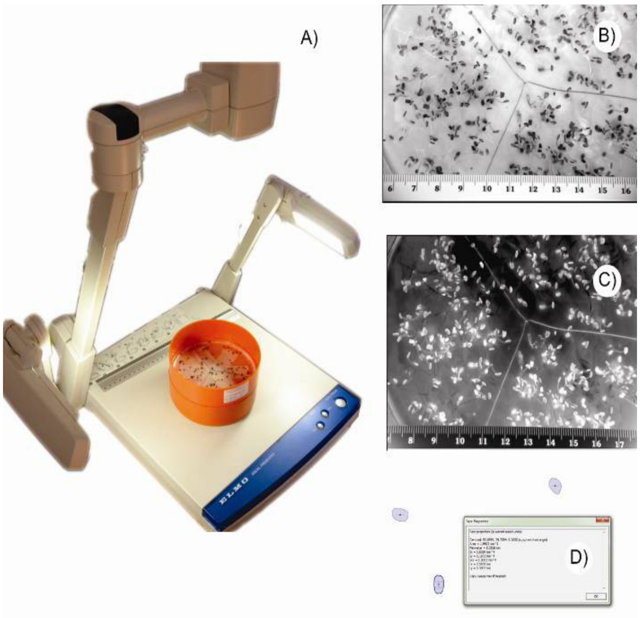

Fig. 2. Digital seed development measurements: A - visual presenter with HD cam; B - black and white photo enhancement; C - positive/negative photo philter enhancement; D - Face Centroid and Area Properties free plug-in (original)

conditions: relative air humidity (45-55%), green house temperature (20-24 °C), light intensity (8,000-9,500 lux).

Data observation: average weight of 1,000 seeds

Before the experimental design, all the received seeds where calculated under the incidence of average weight of 1.000 seeds as follows:

$$\text{Average weight of 1,000 seeds} = \frac{M}{n} 1000 \text{ (g)}$$

M = mass of sample seeds

n = number of seeds

For clarity reasons all the obtained results were compared to Kew seed information data base.

Seed germination data observation

The number of germinated seeds was recorded daily. The germination development was observed using a digital visual presenter equipped with a photo camera linked to a computer. The photo sequencing was made daily, when positive/negative pictures where taken. After the photo capture, all the images where imported in vector enhancing software that generated scale data bases like: seed area, seed perimeter, seed development on x and y radius, radicele length, hypocotyl lenght (Fig. 2).

Seed area, seed perimeter, seed development on x and y radius and radicele length where observed in dynamic on 5 days interval using Face Centroid and Area Properties free plug in (Schreyer, 2015).

The germination percentage was calculated on the basis of 25 seeds per replicate, resulting 100 seeds per treatment.

Germination velocity index (GVI) was observed and determined based on the model of Maguire (1962) as follows:

$$GVI = G_1/N_1 + G_2/N_2 + ... + Gn/Nn$$

where: $G_1, G_2 ... Gn$ = daily number of germinated seeds;

$N_1, N_2 ... Nn$ = number of days from sowing to last count.

Mean germination time (MGT) was evaluated using the methodology proposed by Ellis and Roberts (1981), with the final results expressed in days:

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

where:

t_i - time from the start of the experiment to the i^{th} observation (day for the example);

n_i - number of seeds germinated in the i^{th} time (not the accumulated number, but the number correspondent to the i^{th} observation);

k - last day of germination.

At the end of the experiment, seedling vigour index (SVI) was calculated using Abdul-Baki and Anderson (1973) formula:

SVI = Germination (%) × [Average root length (mm) + Average hypocotyl length (mm)]

The average length of seedling root was measured from the root tip to the insertion point of the hypocotyl. The mean hypocotyl length was measured from the base point to the insertion point of the cotyledon leaves.

Chemical analysis

Fresh basil leaves were collected from each plant and sliced (about 2 mm/slice). In a conical flask, 1.5 g of sample leaves where placed with a mixture of methanol - n-hexane (2:1 v/v). The solvent extraction method was done based on polarity issues regarding flavour compounds. In order to obtain a good exchange capacity between the analyzed material and the solvent, a stirrer was used for 10 min. The final stages of extraction where made by ultrasonic extraction method (USE) for 15 min. The maximum temperature reached at the end of extraction was less than 50 °C. The extracted liquid was separated from the residual plant material by filtration and concentration to 1 mL under vacuum in a rotating evaporator. 1 μL of the obtained extract was injected into a gas chromatograph detector equipped with a capillary column HP-5MS of 30 m nominal length, nominal diameter of 0.25 mm and 0.25 μm nominal film thickness. The software analysis tool was MSD ChemStation. The temperature applied regime was: injector temperature -250 °C; MS quadruple temperature -200 °C; MS source temperature -230 °C. At the oven column were applied three temperature regime ramps as follows: the initial furnace temperature -40 °C kept constant for 5 min, followed by a linear temperature increase of 2 °C/min till 100 °C, maintained constant for 1 min, and then followed by a linear increase of 7 °C/min till 200 °C, maintained constant for 1 min, followed by a linear increase of 10 °C/min till 270 °C, maintained constant for 5 min. The analysis module was full scan.

The compounds were identified using the NIST database by comparing the obtained mass with the spectral mass libraries.

Statistical analysis

The statistical analysis was performed using the Excel 2002 statistical software. The post hoc Duncan test was done using DSAASTAT free VBA Macro. All the charts and graphics where made using multiple interactions between InkScape and SketchUP.

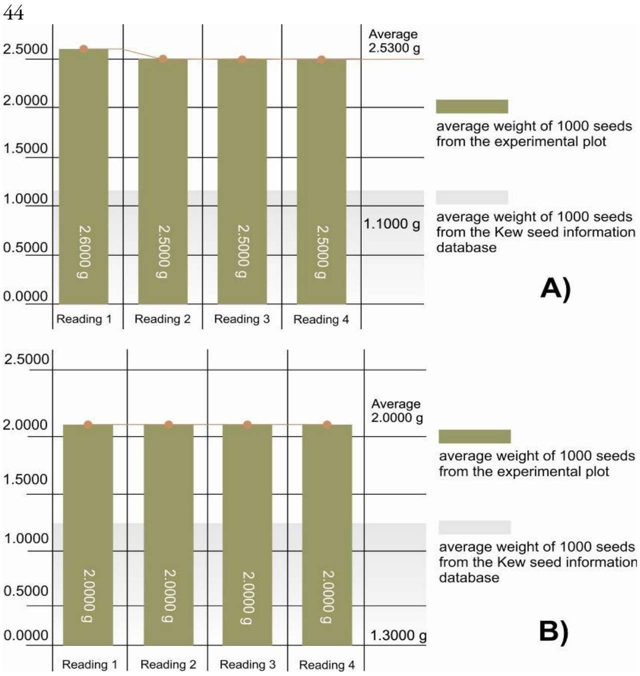

Fig. 3. *Ocimum basilicum* L. (A) and *O. basilicum* var. *purpurascens* Benth. (B) average weight of 1,000 seeds parallel to Kew seed information database (original)

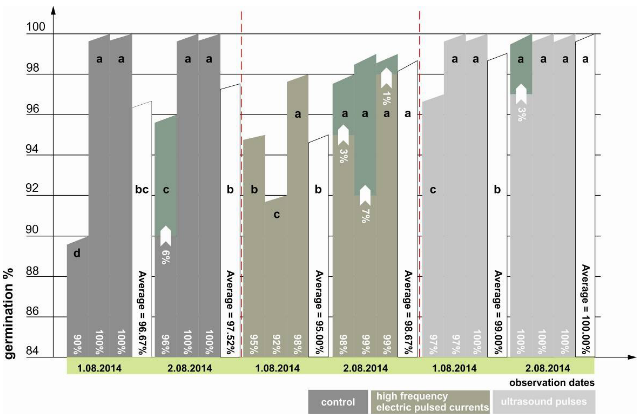

Fig. 4. *O. basilicum* L. germination percent (original). Note: Values followed by different letters indicate significant differences ($p<0.05$) between experimental factors according to Duncan's multiple range test

Results and Discussion

Average weight of 1,000 seeds was made prior to experimental plot design in order to obviate poor germination capacity due to improper seed weight. The Kew seed information database Kew (2016) was interrogated as etalon, obtaining substantial information regarding the weight of 1,000 seeds with an average value of 1.1000 g for *O. basilicum* L. and 1.3000 g for *O. basilicum* var. *purpurascens* Benth.

The average weight of 1,000 seeds from the experimental plot recorded the value of 2.5300 g for *O. basilicum* L., thus 43.5% higher than the standard Kew seed information database and 2.0000 g for *O. basilicum* var. *purpurascens* Benth. 65.00% higher than the same seed database (Fig. 3).

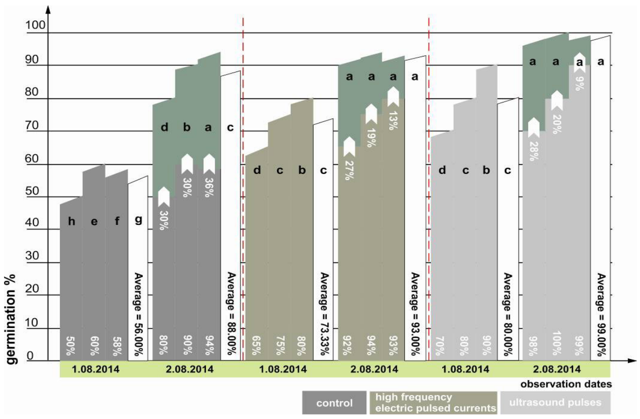

Fig. 5. *Ocimum basilicum* var. *purpurascens* Benth. germination percent (original). Note: Values followed by different letters indicate significant differences ($p<0.05$) between experimental factors according to Duncan's multiple range test

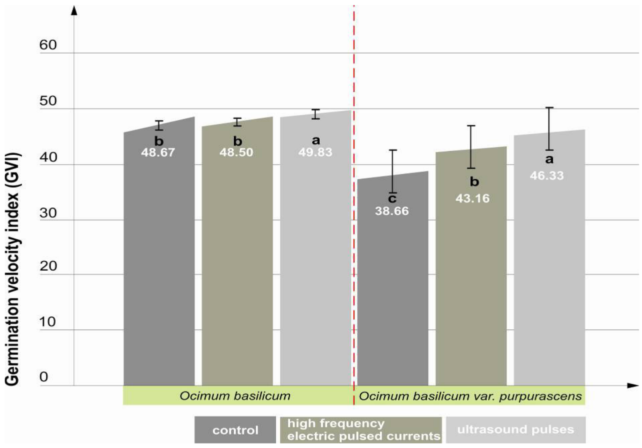

Fig. 6. Germination velocity index (GVI) at *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. (original). Note: Values followed by different letters indicate significant differences ($p<0.05$) between experimental factors according to Duncan's multiple range test

Seed area development was observed with the help of a visual presenter equipped with a HD camera, assuring proper observation at every seed stage development by photo sequencing and raster to vector enhancement.

The germination percent was recorded by confronting the main evaluation criteria - the presence of a radicle of at least 2 mm. All the experimental variants revealed an increased germination percent. The average germination percent was 97.5% for the control experimental variants, 98.7% for the high frequency pulsatile electromagnetic fields, and 100% for the ultrasound pulses experimental variant. Even though ultrasound pulses experimental variants obtained the maximum germination capacity, the results were not assured from the statistical point of view (Fig. 4). During the observation time, germination dynamic ranked from 1% to 7%.

Directly related dynamic was observed for *O. basilicum* var. *purpurascens* Benth. experimental variants with the difference of statistically assured results. Ultrasound pulses experimental variants obtained a significant statistical value,

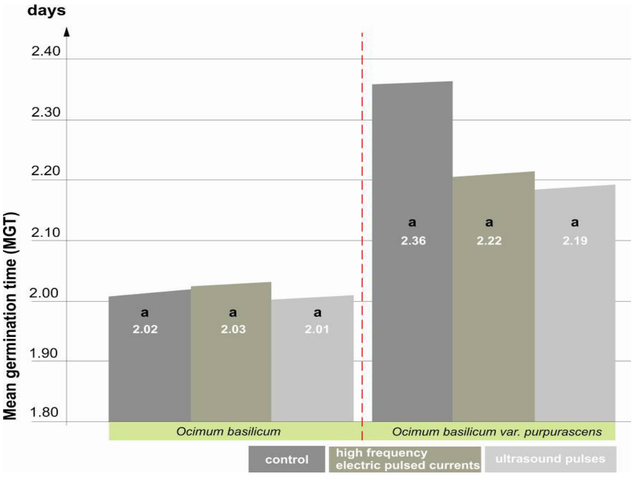

Fig. 7. Mean germination time (MGT) at *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. (original). Note: Values followed by different letters indicate significant differences (p<0.05) between experimental factors according to Duncan's multiple range test

with an average germination of 99.0%. High frequency electric pulsed currents obtain an average germination percent of 93.0%, followed by the control experimental variants with 88.0% (Fig. 5).

Regarding the germination amplitude for *O. basilicum* var. *purpurascens* Benth., it was observed a high difference between the two observation dates ranging between 9% till 36%. It can be deducted from this result that the experimental variants with minimum dynamic percentage between observation dates assured the optimum germination condition.

Even though *O. basilicum* L. experimental variants did not record statistical significance, overall ultrasound pulses experimental variants recorded the maximum germination percent in both cases.

The germination velocity index (GVI) was observed daily by photo sequencing when the number of germinated seeds was recorded and digitally measured. All the experimental variants based on ultrasound pulses obtained a significant statistical value. The second values in range where obtained by the high frequency electric pulsed currents (Fig. 6).

Mean germination time (MGT) sustained the general theory of seed germination that enounced a diametric position between values of GVI and MGT, meaning that all the experimental variants with high values of GVI were keen on recording the lowest values of MGT. The general enounced theory is sustained by the obtained results, where as all the control experimental variants recorded the highest MGT values: 2.02 days for *O. basilicum* L. control experimental variants and 2.36 days for *O. basilicum* var. *purpurascens* Benth. (Fig. 7).

At the end of the experiment, seedling vigour index (SVI) was calculated from a randomized extraction of 6 seedlings per experimental variant. With the help of Face Centroid and Area Properties free plug in, the seedlings where divided by main formula components: seedling root, hypocotyl, cotyledonal leaves (Fig. 8). All seedlings where transformed from raster pictures in vector formats and delimitation colors where applied.

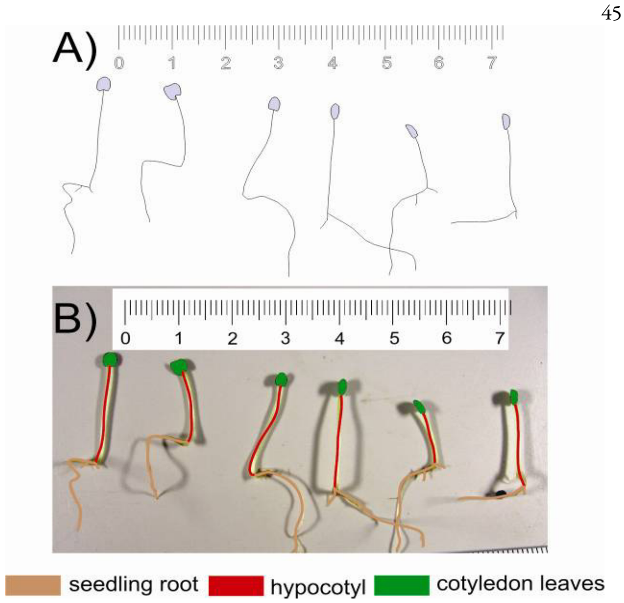

Fig. 8. Seedling vigour index (SVI) calculation method: A - Face Centroid and Area Properties free plug in; B - vector enhancement of seedlings and color associations (original)

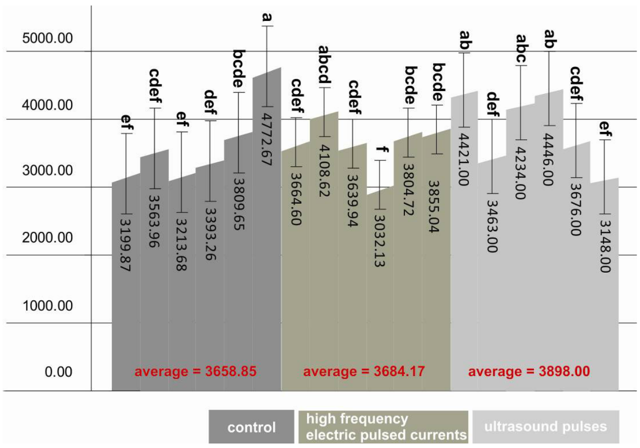

Fig. 9. Seedling vigour index (SVI) of *O. basilicum* L. (original). Note: Values followed by different letters indicate significant differences (p<0.05) between experimental factors according to Duncan's multiple range test

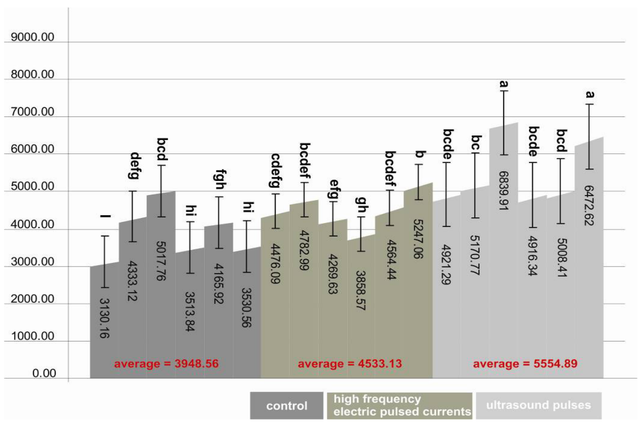

Fig. 10. Seedling vigour index (SVI) of *O. basilicum* var. *purpurascens* Benth. (original). Note: Values followed by different letters indicate significant differences (p<0.05) between experimental factors according to Duncan's multiple range test

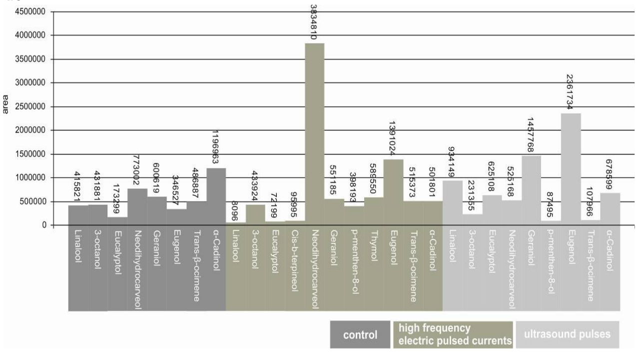

Fig. 11. Average area content of main therapeutical terpenes at *O. basilicum* L. (original)

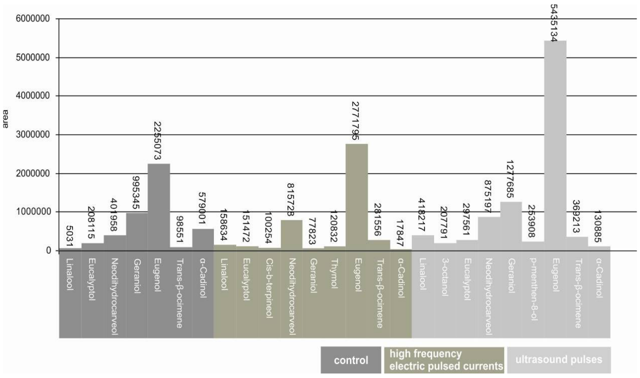

Fig. 12. Average area content of main therapeutical terpenes at *O. basilicum* var. *purpurascens* Benth. (original)

For all tested ultrasound pulses within experimental variants, the seedling vigor index recorded the highest values (3,898.00) for *O. basilicum* L. and 5,554.89 for *O. basilicum* var. *purpurascens* Benth. (Fig. 9 and 10).

The resulting seedlings where transferred in vegetation pots at the green house of Ornamental Plants Department, USAMV Cluj-Napoca, Romania, when they received standard vegetation conditions: relative air humidity (45-55%), green house temperature (20-24 °C), light intensity (8,000-9,500 lux). When young plants formed an average of 10-20 new leaves, quality aspects as main therapeutically terpenes where determined. Prior to gas chromatograph determination, soluble dry substance was determined for each experimental variant at 20 °C. Ultrasound pulses obtained an average reading of 7.6% soluble dry substance, followed by ultrasound pulses (5.4%) and control (5%). Following this solid foundation, the highest content of terpenes was observed in ultrasound pulses and high frequency electric pulsed currents variants (Fig. 11 and 12).

All the experimental variants influenced by physical stimulants recorded improved contents of therapeutical terpenes. In the case of high frequency electric pulsed currents, the main spectrum of therapeutically terpenes was improved by the presence of Cis-β-terpineol and p-menthen-8-ol. The same terpenes improvement was observe at ultrasound pulses experimental variants. *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. recorded the same dynamic in terpenes content for the same experimental variants.

Conclusions

Regarding the *O. basilicum* L. and *O. basilicum* var. *purpurascens* Benth. seeds, ultrasound pulses experimental variants where consider as optimum species for seed germination improvement. A visual statement was observed at the beginning of the experiment, when seeds exposed to ultrasound pulses generated faster secretion of mucilage, a specific pectin solution which allows easier germination and increases the seed's "stickiness" to the soil.

All the experimental variants subjected to different treatments of electromagnetic pulse were influenced by physical stimulants and recorded improved contents of therapeutical terpenes. Mean germination time (MGT) was in agreement with the accepted theory of seed germination that enounced a diametric position between values of germination velocity index (GVI) and MGT, meaning that all the experimental variants with high values of GVI recorded low values of MGT.

Acknowledgements

Dr. Rodica Ungur member of 6th Dept. of Medical Specialities acknowledges financial support from 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca, Romania, internal grant No. 27020/50/15.11.2011.

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