© 2018 *RELS*, **p-ISSN:** 0974-4908
Home page: http://rels.comxa.com
e-mail: rel_sci@yahoo.com
Res. Environ. Life Sci.,
11(08) 222-227 (2018)

Publication Info
Paper received: 16.06.18
Revised received: 17.07.18
Paper Accepted: 19.07.18
Category: Original paper

Effects of ultra-high diluted (UHD) potentised homeopathic medicines on pea (*Pisum sativum* L.) - a hydroponics study under plant growth chamber

Abstract

Ultra high dilution is the dilution in which the medicine is diluted to very lower concentrations to reduce the toxicity of the concentrated homeopathic medicine. A plant growth chamber study of ultra-high diluted (UHD) potentised homeopathic medicine on the seed germination, and the growth of pea seedlings was carried out. Experiments were carried out using homoeopathic medicine, Arsenicum album of different potencies like 6C, 30C, 200C on the plant models, pea (var. Arkil).Different growth parameters including germination percentage shoot length, root length-, wet weight, dry weight and biochemical parameters including photosynthetic pigments, total protein content, and all antioxidant enzymes like- Superoxide dismutase(SOD), Catalase(CAT), Guicol Peroxidase(GPX), Ascorbate peroxidase(APX), Polyphenol oxidase (PPO), Glutathione reductase(GR), Dehydroascorbate reductase (DHAR), Root Oxidase (RO), Ascorbic acid, Phenol content were analysed. The role of UHD potentised homeopathic medicines in promoting germination and growth can act as stimulating agents. A significant effect on germination rate was observed in ultra-high diluted (0.1%) homeopathic medicine of 200C potency for pea. In pea growth as well as biochemical parameters were significant in 30Cand 200C potency respectively.

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Keywords

Potentised, homeopathic medicine, Ultra high diluted (UHD), potency, Hydrponiocs, Plant growth chamber

Introduction

India is one of the developing countries with huge populations, rapid economic growth and limited natural resources, therefore facing the massive pressure of ensuring food security. This higher consumption of affluent food items exerts more pressure on the agricultural land demand of the countries (Nath *et al.,* 2015). Homeopathic potentisation is the process consists of highly diluted substances, such as plant or animal extracts or minerals, which are stepwise diluted and vigorously succussed. Now research in homeopathic potencies is of great interest to organic agriculture since this way of treatment relies totally upon natural substances and essential self-regulation principles (Panda *et al.,* 2017). Homeopathy and anthropological medicine are well-known methods of complementary health care. Both medical systems include the use of homeopathic dilutions or potencies as a specific therapeutic intervention (Baumgartner *et al.,* 2004). Applied Homoeopathy Research in agriculture is also finding more attention now a day. The homeopathic medicine (HM) would be a heterogeneous mixture of nano-sized particles (NPs, small particles that measure 1-100 nanometers along at least one dimension) of the source material, with or without the nonspecific presence of nanosilica released during successions of solutions in borosilicate glassware. (Ives *et al.,* 2010).

At ultra-molecular dilutions (beyond the Avogadro limit), the probability of the presence of molecules of the original substance is near to zero. Astonishingly, to our knowledge, nearly all quantitative meta-analyses of randomized clinical trials for specific medical conditions yielded positive evidence for homeopathic treatments compared to placebo. Fundamental research has to develop a spectrum of standardized tools to investigate the mode of action and nature of homeopathic potencies. (Stock-Schröer *et al.*, 2009). However, for the last few years our research was focused not only on understanding the mechanism(s) of homeopathic action in medical and veterinary practices but also on the effect of different homeopathic preparations on plants. The studies aimed to rule out the placebo effect from the homeopathic phenomenon, but also at the identification of therapeutic effect on separate plants. These studies allowed to extend the boundaries of the practical use of homeopathy including its frequent use in the farming industry. The efficacy of various homeopathic preparations was assessed for their use in plant growth through application techniques such as watering, spraying, soaking and sprinkling etc. Growth dynamics, duration of fruit ripening, crop

yield were monitored and compared to reference plants (Novosadyuk *et al.,* 2015). Homeopathic preparations for agricultural plants were carefully selected on the basis of their effect on the crop yield - the principal criterion for the selection. A selected range of homeopathy medicines is proving to be best bio-fertilizers with pest control properties for many plants and crops during field studies conducted by a volunteer team at the Government Homoeopathic Medical College in Thiruvananthapuram, India (The Hindu, 2001). The results of the "clinical trials" on plants and crops have been much more than impressive, with the application of the liquid form of the drugs in adjusted dosage returning good harvests and enhancing self-protective mechanisms to ward off disease and repel pests and insect attacks (The Hindu, 2001). Healthy plant model seems a useful approach to investigate fundamental research questions about the specificity and efficacy of homeopathic preparations (Baumgartner *et al*., 2004). To evidence the efficacy of homeopathic medicines in the treatment of diseases and the effectiveness of homeopathic dilutions in biological systems, clinical and experimental studies are conducted with human beings, animals, plants, cell cultures, etc. (Teixeira *et al.,* 2017).

In this present investigation, the effect of different concentration of ultra-high diluted homeopathic medicine (6C, 30C, 200C) on *Pisum sativum* (L.) plant models have checked.

Material and Methods

Potentised homeopathic medicine: Homeopathic potencies are based on successive logarithmic dilution steps (1:10 for X (D) potencies, 1:100 for Centesimal (C) potencies, some of which end up as quite high dilution ratios. The potentised homeopathic medicines are diluted to different concentrations like 1%, 0.5%, 0.1%, and 0.05% for the experiment.

Experimental design: Pea seeds (Arkil) were purchased from Govind Ballabh Pant AgricultureUniversity, Uttarakhand for the experiment**.** Potentised homeopathic medicine *Arsenicum album* were diluted *for* four separate experiments as control and 6C, 30C, 200C for physiological and biochemical analysis . Plants are grown from germination to till the end of the experiment in plant growth chamber (Make- B.P. Lab Solutions). Rate of germination was calculated by treating the seeds with different ultra-high diluted potentised solution (1-0.05%). Germination rate was maximum in the ultra-diluted homeopathic medicine (0.1%) compared to other dilutions. Then the germinated seeds were grown on both hydroponics, pot culture for various physiological and biochemical assay.

Germination: The rate of germination varies in different ultrahigh diluted potency of homeopathy medicine (1-0.05%) in case of pea (Arkil) seeds. This indicates that this ultra-diluted homeopathic medicine has stimulatory effect on the germination as compared to control or untreated. Seeds of uniform in size; colour and weight were chosen for experimental purpose. Petridish culture was used to carry out for germination test. Uniform sized seeds of pea was surface sterilized using 0.01% $HgCl_2$ and then allowed to germinate on cotton pad saturated with appropriate treatment solution. The seeds were allowed to germinate in different ultra-high diluted homeopathic medicine (1-0.05%) under controlled condition at 25°C in darkness for two days. A seed was considered as normally germinated when the radicle protruded by 2-3 cm without infection and the first leaves were visible (ISTA, 1999). The experiment was carried out in three replicates with completely randomized block design. Rate of germination was recorded on the 5th day and the percentage was calculated using below formula:

% of germination= (No. of germination× 100)/ Total no. of seeds sown

Hydroponics Culture: After germination, seeds were randomly selected and transferred to well aerated Hoagland's nutrient solution (full strength) and Hoagland's solution supplemented with ultra-diluted potency of homeopathic medicine in hydroponics culture vessels for seedling growth (Arnon and Stout, 1949). The seedlings were grown in the growth chamber and the white light was provided (12h photo period) by white fluorescent tubes(36 W Philips TLD) with a photon flux density of 52 $\mu Em^{-2} S^{-1}$(PAR). The nutrient solution was aerated twice a day, and changed three times in a week. After two weeks (on 15th day), various growth parameters ,photosynthetic pigments and biochemical parameters were analysed.

Growth parameters: Growth was assessed in terms of length of the shoots and roots, fresh weights and dry weight of 15and 21-day-old seedlings of pea. Seedlings were washed thoroughly with distilled water and soaked by using blotting papers. Roots and shoots were first detached from each other. Individual length of root and shoot were measured in centimetre scale. Fresh matter content of both control and treated samples were noted with the help of electric balance. These seedlings were then kept in oven at 80^0C for a period of three days or more for dry weight determination. Then constant dry weight of root and shoot biomass is measured.

Analysis of pigments: Photosynthesis is the most important biochemical event on earth. It serves as the world's largest solar battery. Photosynthesis converts massive amount of sunlight into electrical and then chemical energy (Hall and Rao, 1999). The most important photosynthetic pigment is chloroplast consists of two types of chlorophylls, chlorophyll-a and chlorophyll-b. Chemically they differ from each other and absorb light of different wavelength to perform photosynthesis.

Photosynthetic pigments like chlorophyll a, chlorophyll b and total chlorophyll were measured using the equation adopted by Porra *et al.,* 2002:

Chlorophyll a (µg chl. a/ml) $=12.25(A_{663.3})-2.55(A_{645.6})$

Chlorophyll b (µg chl. b/ml) $=20.31(A_{645.6})-4.91(A_{663.3})$

Where, A= Absorbency at specific wavelength

Other pigments like pheophytin-a, pheophytin-b, total pheophytin, and total carotenoid were measured using the following equation adopted by Litchender HK, 1987.

Estimation of soluble protein: The soluble protein was estimated using the standard protocol (Lowry *et al.,*1951).According to which, fresh leaf sample were homogenized in 10.0 ml of prechilled 0.1 M phosphate buffer (pH 7.0) under ice cold conditions. The homogenate was ûltered and centrifuged at 12,000 g for 10 min. The supernatant was stored at 4°C until used for assaying activities.

Table-2: Physical conditions maintained in plant growth chamber during pea (*Pisum sativum L.*) growth under hydroponics

Day Time	Temperature	Light Level (incandescent: Fluorescent)	Light Intensity (μ E m-2 s-1)
9.00 am	230C	1:0	76
9.15 am	24^{0}C	1:2	280
10.00 am	32^{0}C	0:4	625
1.00 pm	34^{0}C	0:6	898
4.00 pm	32^{0}C	0:6	898
6.00 pm	34^{0}C	0:4	625
6.15 pm	34^{0}C	1:1	130
6.30 pm	28^{0}C	0:0	0

*Humidity at day time was 53% and at night it was 67%
*Airflow from bottom to top (vertical current) was 14 meters per minute

An aliquot (0.5 ml) of the supernatant was used for protein estimation, using bovine serum albumin as a calibration standard.

Enzymes assays: Leaf sample (100 mg) was extracted with 5.0 ml of TCA (0.1%, w/v) in an ice bath, and the homogenate was centrifuged at 12,000 g for 15 min for Peroxidase (POX),(Velikovaet *et al.,*2000). To 0.5 ml of the supernatant, 0.5 ml of phosphate buffer (pH 7.0) and 1.0 ml of potassium iodide (1 M) were added. The absorbance of the mixture was measured at 390 nm. H_2O_2 content was determined using an extinction coefficient of 0.28 µM $^{-1}$cm $^{-1}$ and expressed as n mol per g.

Ascorbate peroxidase was measured following Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290nm (E = 2.8 mmol [cm^{-1}). The reaction mixture (3 ml) contained 50 mM Na_2PO_4 buffer of pH 7.0 (1.8 ml), 100 mM H_2O_2 (0.5 ml) and 0.5 mM ascorbic acid (0.5 ml) and 0.2 ml of enzyme aliquot. Their assay is an essential aspect in assessing stress responses in plants.

Superoxide dismutase was measured by the photochemical method described by Gianopolitis and Ries (1977) with modifications suggested by Choudhury and Choudhury (1985). Three ml reaction mixture contained 2.4 ml (50 mM) Na_2PO_4 buffer (pH 7.8) containing 0.1 mM EDTA, 63 µM nitro blue tetrazolium chloride (NBT) and 13 µM L-methionine, 0.2 ml of enzyme extract and 0.5 ml riboflavin (1.3µM). Riboflavin was added last. The reaction was monitored in the presence of two 40 V fluorescent lamp for twenty min. The distance between reaction tubes and lamps was 30 cm. One unit of SOD activity was defined as the amount of enzymes required to cause 50 % inhibition of the rate of nitro blue tetrazolium chloride reduction at 560 nm and was expressed unit g^{-1} fresh weight.

Ascorbate peroxidase was measured following Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290nm (E = 2.8 mmol cm^{-1}). The reaction mixture (3 ml) contained 50 mM Na_2PO_4 buffer of pH 7.0 (1.8 ml), 100 mM H_2O_2 (0.5 ml) and 0.5 mM ascorbic acid (0.5 ml) and 0.2 ml of enzyme aliquot. The reaction was monitored for 3 min and activity was expressed as mM ascorbate oxidised min^{-1} g^{-1} fresh weight.

Catalase activity was measured following Cakmak and Marschner (1992) in a reaction mixture (3 ml) containing 50 mM Na_2PO_4 buffer pH 7.0 (2.3 ml), 10 mM H_2O_2 (0.5 ml)and the 0.2 ml enzyme extract. The decomposition of $H_2O_2$was monitored at 240 nm for 3 min. The activity was expressed as change in OD min^{-1} g^{-1} fresh weight.

The activity of guaiacol peroxidase was assayed following the method of Rao *et al.,*1995. The reaction mixture contained 50 mM Na_2PO_4 buffer pH 7.0 (1.8 ml), 0.1 mM guaiacol (0.5 ml) and 0.1 mM H_2O_2 (0.5 ml). Enzyme activity was measured by the increase in absorbance at 470 nm caused by guaiacol oxidation (E = 26.6 mM cm^{-1}) and activity was expressed in mM guaiacol oxidised min^{-1} g^{-1} fresh weight.

Polyphenol oxidase was measured following Sarkar *et al.,* 2001, by monitoring the change in colour intensity by oxidation of catechol at 420 nm. The reaction mixture contained 50 mM Na_2PO_4 buffer pH 7.0 (1.8 ml), 0.5 % (w/v) catechol (0.5 ml) and 0.1mM H_2O_2 (0.5 ml). Enzyme activity was measured by the increase in absorbance at 420 nm caused by catechol oxidation and expressed as change in OD min^{-1} g^{-1} fresh weight.

Glutathione reductase was assayed according to the method of Foyer and Halliwell (1976) by following the decrease in absorbance at 340 nm caused by NADPH oxidation (E = 6.2 mM cm^{-1}). The 3 ml reaction mixture contained 1.8 ml potassium phosphate buffer (50 mM, pH 7.0) with 1 mM EDTA, 0.5 ml oxidized glutathione (0.5 mM), 0.5 ml NADH (0.2 mM) and 0.2 ml of enzyme extract. The activity of the enzymes was measured by decrease in absorbance at 340 nm starting from 0 to 3 min with 1 min intervals and expressed as mM glutathione oxidised min^{-1} g^{-1} fresh weight.

Dehydroascorbate reductase was measured by following Nakano and Asada (1981) by increase in absorbance at 265 nm. The 3 ml reaction mixture contained 1.8 ml potassium phosphate buffer (50 mM, pH 7.0) with 1 mM EDTA, 0.5 ml reduced glutathione (2.5 mM), 0.5 ml dehydroascorbic acid (0.2 mM) and 0.2 ml of enzyme extract. The activity of the enzymes was measured by increase in absorbance at 265 nm starting from 0 to 3 min with 1 min intervals and expressed as change in OD min^{-1} g^{-1} fresh weight.

The oxidized activity of rice root was measured following the method of Ota, (1970). Roots were washed thoroughly with distilled water and moisture of the roots were removed by blotting paper and cut into small pieces. One-gram fresh root was immediately transformed into 150 ml conical flask containing 50 ml napthylamine (20 ppm) along with one control for 2 h with constant shaking. Two ml aliquot was transformed to a graduated test tube and diluted to 10 ml with distilled water. One-ml of Sodium nitrite (100 ppm) and 1 ml of 1 % sulphonilic acid (in 30 % acetic acid) was added with shaking to each tube. The volume was made up to 20 ml. After 30 min the intensity of pink colour was measured at 500 nm. The oxidized napthylamine quantity was calculated per unit fresh weight of roots per unit time by taking the difference between the control and sample.

Ascorbic acid content was determined following Sigeoka *et al.,*1979. Fresh leaf sample weighing 500 mg was homogenized in 3 ml 5 % (w/v) metaphosphoric acid. The extract was clarified by centrifugation at 2000 g for 20 min at 4^{o}C and the supernatant was used for the assay. An aliquot measuring 0.5 ml was mixed with 0.25 ml of 3 mM DCPIP to measure the total ascorbic acid content whereas an equal volume of water was added when the oxidized

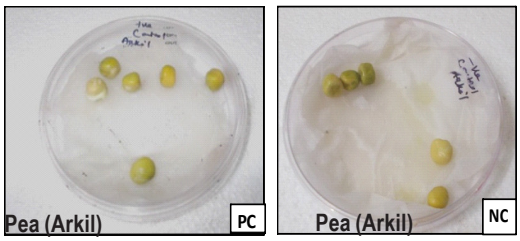

Fig. 1: Pea (Arkil) seed germination photo

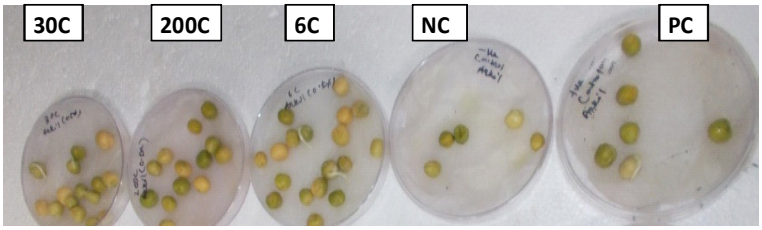

Fig-2: Different potency homoeopathic medicines treated pea seed germination photo

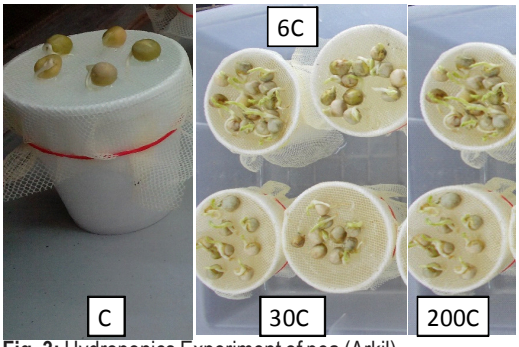

Fig. 3: Hydroponics Experiment of pea (Arkil)

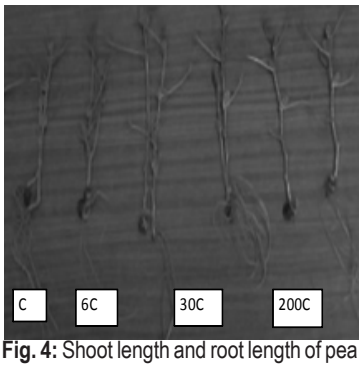

Fig. 4: Shoot length and root length of pea

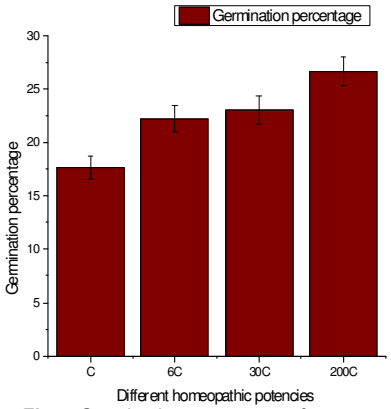

Fig.5: Germination percentage of pea

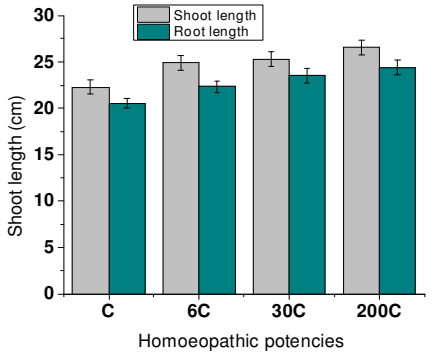

Fig.6: Shoot length and root length of Pea after15 days

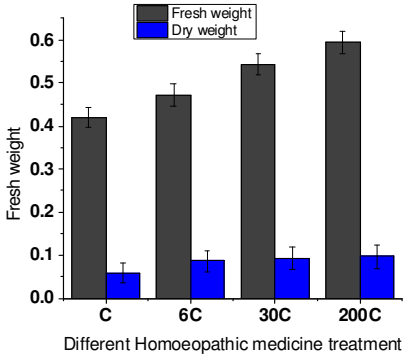

Fig.7: Fresh wt. and dry wt. of Pea after 15 days

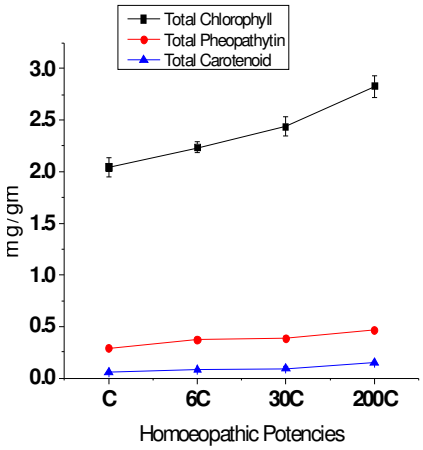

Fig.8: Photosynthetic pigments of Pea

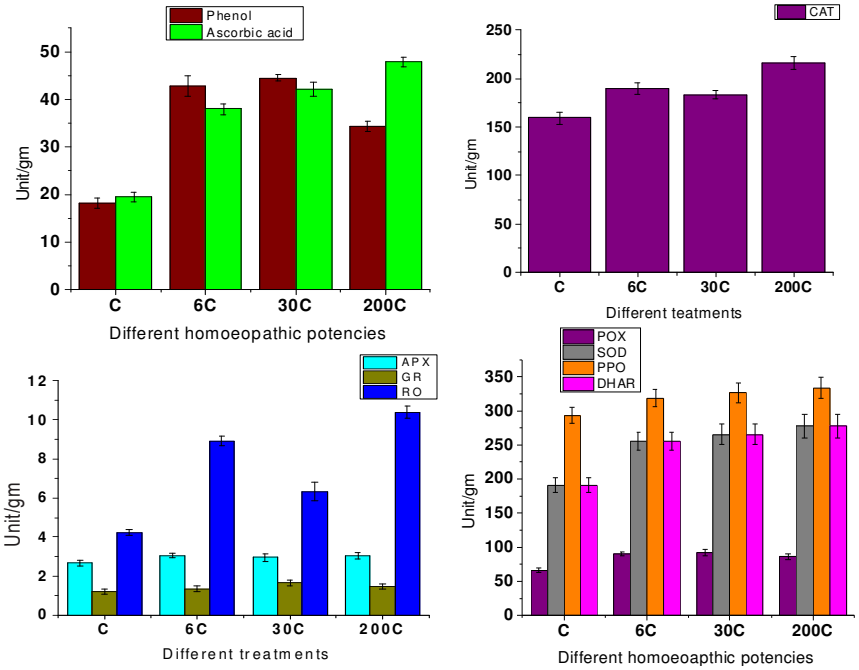

Fig. 9: Antioxidant enzymes analysis of Pea (Arkil) after 15 days

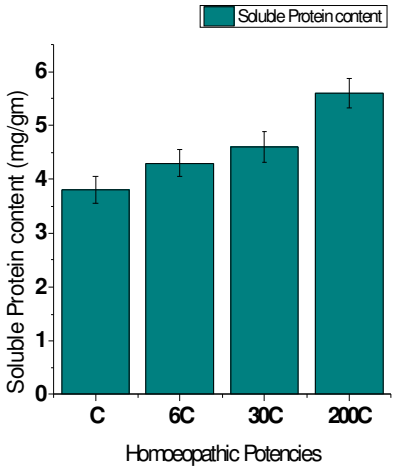

Fig.10: Total soluble protein contents of pea

ascorbic acid content was to be measured. After keeping the mixture at room temperature for 20 min 0.5 ml of 1% (w/v) thiourea in 5 % (w/v) meta-phosphoric acid and 0.5 ml of DNPH was added. The mixture was then incubated at 50 ºC for 1 h, cooled in an ice bath for 15 min while adding 1.2 ml of ice cold 85 % H_2SO_4. Then absorbance at 520 nm was recorded. The amount of reduced ascorbate was calculated by subtracting oxidized ascorbate content from total ascorbate.

Total phenol content was measured following the method of Sadasivum and Manickum (1997). Fresh leaf tissue of 0.5 g was homogenized in 10 ml of 80 % ethanol, clarified by centrifuge at 10,000 rpm for 20 min. The supernatant was evaporated to dryness, and dissolved the residue with 5 ml of distilled water. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of FC reagent, after incubation for 3 min 2 ml of 20 % Na_2CO_3 solution was added. The absorbance was taken at 650 nm against a reagent blank. Phenol content was expressed as mg Phenol g^{-1} fresh weight using catechol as standard.

Statistical Analysis: Statistical analysis One way ANOVA was carried out for Pea (Arkil). One way ANOVA analysis was carried out by using the Origin Pro 9 software. The significance level was analysed at 0.05 level. All statistical calculations like antioxidant enzymes, photosynthetic pigments, protein, shoot length, root length, fresh weight and dry weight have been analysed. At significant at 0.05 level the significance of the experiment were analysed.

Results and Discussion

Germination: A significant effect in on germination rate was observed in 0.1% diluted concentration. 0.1M Calcium carbonate was applied at the time of germination. In Pea (Arkil) germination rate in potentised homoeopathic medicine 200C was significant than the other treatments was observed. The result was somehow contradictory as pervious observations which were done by some scientific communities(Ganger HU 2007, Brizzi M, Betti L 2010).

Growth Parameters: The morphological parameters like shoot, root length and fresh, dry weight was greatly influenced in both wheat and pea seedlings by the application of the homeopathic solution. It was observed that different plants responded differently to the ultra-diluted homeopathic medicine (0.1%) of the same homeopathic drug.

The pea seedlings show with increase in the ultra-diluted homeopathy medicine (0.1%) potency, the average length of the shoot and root length increases. The trend was also similar to the fresh and dry mass of the seedlings. But in case of wheat, the trend was different and at potentised homeopathic medicine 200C the stimulatory effect was maximum. Similar experimental findings were reported by Baumgartner *et al.,*1998.

Photosynthetic pigments: Photosynthetic pigment estimation is one of the most important biochemical parameters which were used as the index of production capacity. Fig. 8 concludes that the pigment contents in plants showed an almost linear increase in response to increase in potency of homeopathic drugs.

Pigments like- chlorophyll-a, b and total chlorophyll increased with increase in the potency of the medicine. Total chlorophyll content was highest in *Arsenicum album* at 200C potency. Other photosynthetic pigments like-carotenoid, phaeophytin -a, b, and total phaeophytin shows a positive effect on when treated with homeopathic medicines (Fig. 8). The result indicates that homeopathic drugs at higher potency had a significant stimulatory effect.

Photosynthetic pigments like total chlorophyll, total pheophytin, and total carotenoid were analysed in Pea (Arkil). It was observed that Pea show significant result in 200C potency.

Antioxidant Enzyme: All antioxidant enzymes like-Superoxide dismutase(SOD) Catalase(CAT), Guicol Peroxidase (GPX), Ascorbate peroxidase (APX), Polyphenol oxidase (PPO), Glutathione reductase (GR), Dehydroascorbate reductase (DHAR), Root Oxidase (RO), Ascorbic acid, Phenol were analysed. In Pea, effects of Ultra-high diluted (0.1%) potentised homeopathic medicine (200C) of *Arsenicum album* showed significant result for all above antioxidant enzymes than other treatments.

Protein: Standardised ultra-high diluted (0.1%) concentration of different potentised homeopathic medicines (6C, 30C, 200C) treatments were used for the experiment work. In lowest potency (6C) the soluble protein content was reduced and increased with the increase in homeopathic potencies concentration. It was observed that 200C potency show the significant soluble protein content in pea, than the other potencies like - 6C and 30C.

After One way ANOVA statistical analysis of all experiments it may be concluded that the ultra-high diluted (0.1%) potentised homeopathic medicine 30C shows the best results in morphological and biochemical responses of wheat plants but in pea 200C shows the significant result than control and other treatments. One way ANOVA statistical analysis have been conducted and the significance of experimental data were analyzed at 0.05 significant level. Physiological and biochemical responses of plants to ultra-high diluted potentised homoeopathic medicine *Arsenicum album* can be viewed as potentially adaptive changes that decline the operation of metabolic regulatory mechanisms which favors the functioning of the plants during and after stress. Thus the data which is generated through this study will be very helpful in implementing the use of these ultra-dilute drugs in agriculture (Agro homeopathy).

Acknowledgment

The authors are grateful to CCRH, New Delhi funding agency for financial support and the Director, CSIR-IMMT for laboratory facility to carry out the experiments.

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