Original Article

Sepia 200 cH in 1:1,000 dilution counteracts the effect of salt stress in cowpea seedlings but vehicle 90% ethanol proves ineffective in the same dilution

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ABSTRACT

Background: Soil salinity severely affects crop yield all over the world. In a recent study, we found that homeopathic drug *Natrum muriaticum* 200 cH improved growth in germinating cowpea [*Vigna unguiculata* (L.) Walp] seeds. **Aim**: In the present study, we tested homeopathic drug *Sepia succus*, which is complementary to *Nat-m* on cowpea seedlings under salt stress. **Methods**: Cowpea seedlings grown over moist filter paper in Petri dishes were divided in 4 groups: (1) control in sterile water, (2) in 50 mM NaCl solution, (3) seeds pretreated with 90% ethanol diluted with water 1:100 and then transferred to 50 mM NaCl solution, and (4) seeds pretreated with *Sep* 200 cH diluted with water 1:100 and transferred to 50 mM NaCl solution. In another experiment the groups were same, but the dilution of 90% ethanol and *Sep* 200 cH was 1:1,000 instead of 1:100 to further reduce the ethanol content in both drug and its vehicle 90% ethanol, to minimize or abolish the effect of alcohol. The data were analyzed by ANOVA followed by Student's t-test. **Results**: *Sep* 200 cH in both 1:100 and 1:1,000 dilutions significantly increased the growth, sugar, chlorophyll, protein and water content of seedlings as compared to the untreated salt-stressed group. The effect with the 1000th dilution of *Sep* 200 cH was more pronounced compared to the 100th dilution. Vehicle 90% ethanol in 1:100 dilution produced some positive effects on the seedlings, but its 1000th dilution produced no such effect. **Conclusions**: *Sep* 200 cH counteracted the effects of salt stress in cowpea seedlings, and its 1000th dilution was more effective than the 100th dilution. The effect of alcohol was totally eliminated with the 1000th dilution of 90% ethanol. Therefore, the 1000th dilution could retain the drug effect and eliminate the vehicle effect.

Keywords: *Sepia succus*, salt stress, cowpea seedling, growth, ethanol effect, homeopathy.

Introduction

Salinity is an important abiotic factor that hampers the growth and yield of crops [1, 2]. About 20% of all cultivated lands in the world exhibit high salinity affecting plant growth [1, 3]. Salinity accounts for an annual loss of about USD 12 billion in agricultural productivity [1]. Salt stress is accentuated in areas where the temperature is high and rainfall low [4, 5]. Seedlings are most susceptible to salt stress [6]. No effective agents were found to counteract the effect of salt stress in plants. In a recent study, we have found that *Natrum muriaticum*, a homeopathic drug, could reverse salt stress in germinating cowpea seeds to some extent [7]. In homeopathy, *Nat-m*, which is prepared from sodium chloride, is used for patients with strong desire for salt consumption. According to some authors, homeopathic medicine *Sepia succus* complements the action of *Nat-m* [8, 9].

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The aim of the present study was to establish whether *Sep* could counteract the effects of salt stress in germinating cowpea seeds. Since solution of ethanol in water is used as vehicle of homeopathic drugs, it is also used as control in experimental studies. However, aqueous ethanol exerts effects of its own in a dose-dependent manner. Therefore, the second aim of the present study was to minimize the effect of alcohol by diluting it with water without, however, compromising the actual drug effect.

Sep is prepared from the secretion of the ink sac of cuttlefish (Sepiidae) [9] a marine mollusk. Sepia melanin, obtained from *Sepia officinalis*, is a biopolymer and consists of more than 98% of eumelanin. The color of eumelanin varies from brown to black, and it is insoluble in a broad range of solvents and pH. The metal ions contained by this pigment are Na^{+},K^{+},Ca^{2+}, Mg^{2+} and Fe^{3+} with different polymeric grade chains. Sepia melanin contains phenolic hydroxyl (OH), carboxylic (COOH), and amino (NH) groups as potential binding sites for the metal ions [10].

Materials and methods

Seeds

Seeds of cowpea (*Vigna unguiculata* (L.) Walp), var BC3, were selected based on their uniformity of size and shape. The surface of the seeds was sterilized with 0.1% mercuric chloride [11], washed 10 times with sterile distilled water, and kept to imbibe in sterile distilled water (1:5 w/v) in dark [12] for 17 hours for. The water-soaked seeds were then randomly selected, and their viability was tested using tetrazolium salt [13]. The seeds were obtained from Bidhan Chandra KrishiVisvavidyalaya, Kalyani, West Bengal.

Treatment

Sep 200 cH, purchased from Seth Dey and Co, Kolkata, was diluted with sterile distilled water 1:100 for experiment I, and 1:1,000 for experiment II. A sample of the water-soaked seeds was immersed in the 100^{th} dilution of the drug, and another sample in the 1000^{th} dilution of the drug for 45 min. The two dilutions were not succussed before use. Aqueous ethanol (90%), the vehicle of the drug, was diluted with sterile distilled water in two proportions, 1:100 and 1:1,000, to prepare two dilutions of ethanol similar to the two dilutions of the drug. A sample of 50 mM aqueous solution of sodium chloride was used to induce salt stress in both the experiments. After pretreatment with the drug or 90% ethanol solution, the seeds were transferred to Petri dishes (14 cm x 3 cm), each containing Whatman filter paper grade 1. The Petri dishes were allocated to the following groups (n = 5), each dish containing 40 seeds.

Experiment I

Group 1: (Water) Control with untreated seeds in sterile water.

Group 2: (Salt) Untreated seeds in 50 mM NaCl solution.

Group 3: (Eth H) Seeds pretreated with 90% ethanol diluted with water 1:100 and then transferred to 50 mM NaCl solution.

Group 4: (Sepia H) Seeds pretreated with *Sep* 200 cH (dilution 1:100) and then transferred to 50 mM NaCl solution.

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In experiment II, groups were the same as in experiment I, but the ethanol (Eth T) and *Sep* solutions (Sepia T) were diluted with water in proportion 1:1000 (n=200 seeds/group).

All those treatments were performed under aseptic conditions in laminar flow. The amount of drug or ethanol solution was 10 ml, which was enough to saturate the filter paper in the Petri dishes. The seeds were allowed to germinate in a germinator in room temperature, light and humidity for 48 h in the first stage. Treatment with ethanol solution was repeated once again in groups 3 (Eth H and Eth T), and with *Sep* solutions (Sepia H and Sepia T) in group 4 for 20 min. After the second treatment, the seeds were transferred to a new set of Petri dishes to rule out the possibility of salt deposition. The seedlings were allowed to grow for further 96 hours.

Parameters measured

Morphological

After 144 hours in a germinator at 32 ^{0}C, the seedlings were gently detached from the cotyledons (Figure 1) and their length and weight were measured. They were then placed in an oven at 65 ^{0}C for 72 hours, and weighed again to establish the dry mass. The water content of the seedlings was calculated by subtracting the dry from the fresh weight.

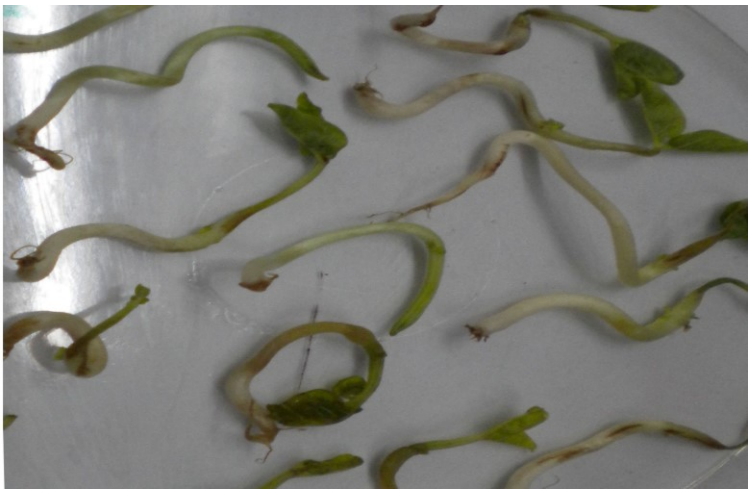

Figure 1. Germinated cowpea seedlings after 144 h without cotyledons.

Biochemical

Chlorophyll estimation was performed by Arnon's method [14], protein estimation by Lowry's method [15], and soluble and insoluble sugars by Anthrone's method [16].

Statistical analysis

All the data were analyzed by means of one-way ANOVA followed by Student's t-test using Microsoft Excel 2007 and SPSS.

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Results

All the data are presented as histograms (figures 2 to 9).

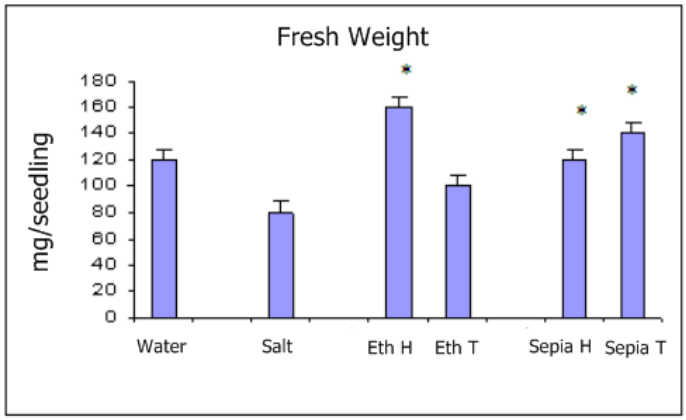
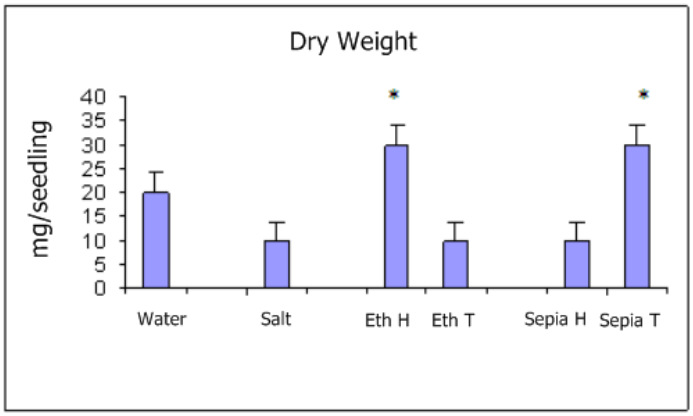


Figure 2. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the fresh weight of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.

Figure 3. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the dry weight of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.



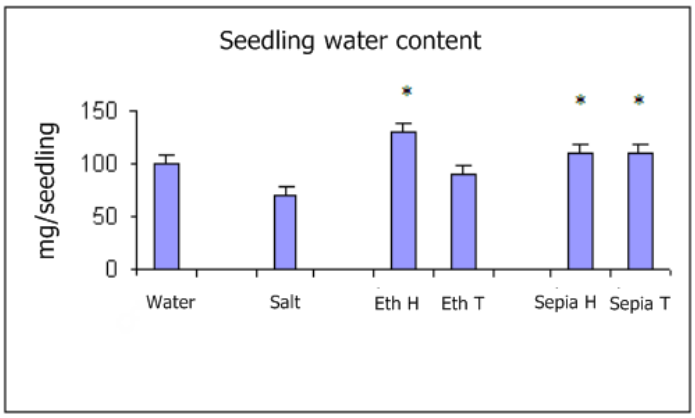


Figure 4. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the water content of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.

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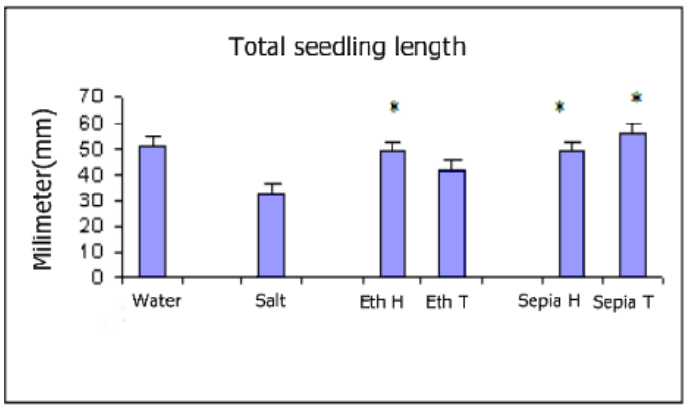
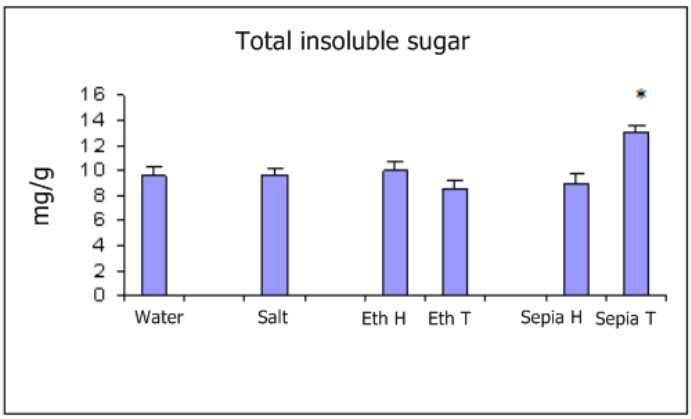


Figure 5. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the length growth of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.

Figure 6. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the insoluble sugar of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.



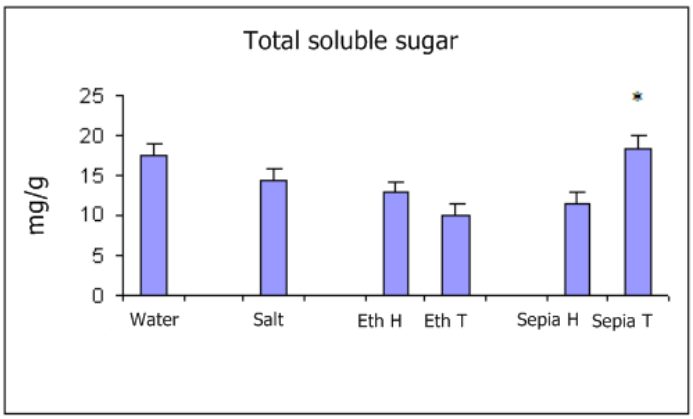
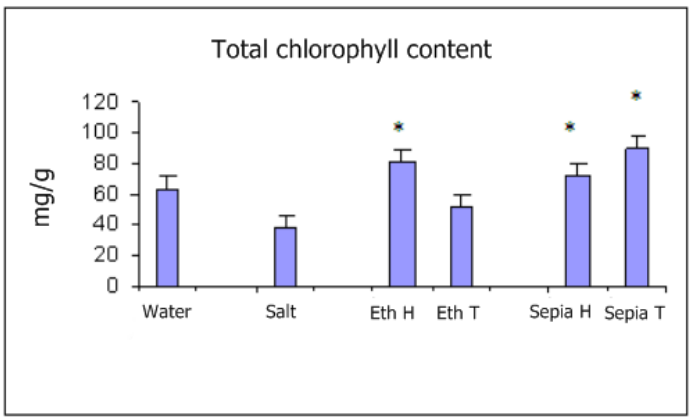


Figure 7. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the soluble sugar of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.

Figure 8. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the chlorophyll content of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.



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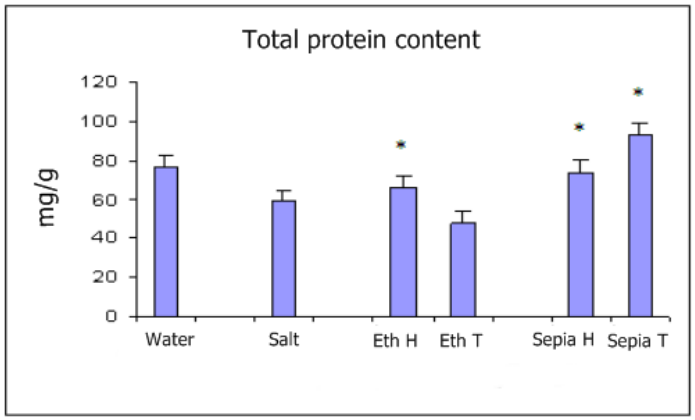

Figure 9. Effect of pretreatment with *Sep* 200 cH and 90% ethanol 1:100 (Sepia H, Eth H) and 1:1,000 (Sepia T, Eth T) dilutions on the total protein of cowpea seedlings grown in 50 mM NaCl solution. *Significant difference ($p < 0.05$) from the untreated salt group. n=200 seeds/group.

Sep 200 cH in 1:1,000 dilution (Sepia T) significantly increased the fresh weight ($p<0.05$), dry weight ($p<0.05$), water content ($p<0.05$), length ($p<0.05$), insoluble sugar ($p<0.05$), soluble sugar ($p<0.05$), chlorophyll content ($p<0.05$) and protein content of seedlings compared to the untreated salt group (Salt) and sterile water control (Water). In all such cases, the control 90% ethanol in 1:1,000 dilution (Eth T) did not show any marked difference from the untreated salt group (Salt).

However, this 1000th dilution of 90% ethanol (Eth T) showed some positive, although not significant effect on the fresh weight of seedlings, and seedling water content compared to the untreated salt group (salt) (Figures 2 to 4). *Sep* 200 cH in 1:100 dilution (Sepia H) significantly increased the fresh weight ($p<0.05$), water content ($p<0.05$), length ($p<0.05$), chlorophyll content ($p<0.05$) and total protein content compared to the untreated salt group (Salt). Here the control 90% ethanol in 1:100 dilution (Eth H) produced significant increase in all those variables compared to the untreated salt group (Salt) (Figures 2, 3, 4, 5, 8 and 9). Except for the insoluble sugar and soluble sugar content, 90% ethanol in 1:100 dilution (Eth H) produced significant increase of the remainder of parameters (Figures 2, 3, 4, 5, 6 and 9) compared to the untreated salt group (Salt).

Discussion

The results indicate very clearly that *Sep* 200 cH effectively mitigated salt stress in germinating cowpea seeds. The most remarkable finding of the present study was that the 1000th dilution of *Sep* 200 cH in water was more effective than the 100th dilution of the drug. Further investigation is necessary to recommend this dilution for therapeutic use among patients.

Long ago researchers observed that the alcohol solution used as control produces some effects, whereby it might be difficult to distinguish the actual effect of a drug from that of its vehicle. The present study showed that the effect of alcohol could be eliminated when both active drug and control ethanol were diluted 1:1,000 in water. This dilution might be recommended for all experimental studies using homeopathic preparations. The alcohol content of the 1000th dilution of 90% alcohol or homeopathic drug prepared in 90% ethanol is 0.09%. In some previous studies conducted with animals, the percentage of alcohol used was 0.3% [17, 18]. In

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some other studies, the drugs were prepared in distilled water, and positive results were obtained [19, 20, 21]. However, the efficacy of aqueous preparations deteriorates over time [22].

Salt stress reduces the total protein content in germinating wheat and cowpeas [23, 24]. Salinity also reduces the K+ concentration, K/Na ratio, seedling length, and total chlorophyll content in different cultivars of cowpea [25]. The water content of the seedlings decreased significantly in the untreated salt-stressed group, whereas treatment with *Sep* 200 cH reversed that effect, and raised the water content to the level of the unstressed control group (Figure 3). The salt solution not only thwarts the entry of water into the seedling tissues due to the high osmotic pressure, but also removes water from the tissues. Water-channel proteins or aquaporins actively transport water in the tissues and block the passage of ions [26]. They are transmembrane proteins that regulate the water flow through membranes during the growth, development, and stress responses of plants [27]. Under adverse conditions, aquaporins are expressed in large amounts or transferred from the interior of the cytoplasm to the cell surface [28]. The homeopathic drug *Sep* might have probably increased the amount of aquaporins on the cell surface of the seedling tissue, thus increasing the transport of water from the external solution into the tissues.

Acknowledgements

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Sepia 200 cH em diluição 1:1.000 reverte o efeito do estresse salino em plântulas de feijão-fradinho, mas o veículo etanol a 90% na mesma diluição é inefetivo

RESUMO

Introdução: A salinidade do solo afeta seriamente a produtividade das culturas no mundo todo. Num estudo recente, observamos que o medicamento homeopático *Natrum muriaticum* 200 cH melhorou o crescimento de sementes de feijão-fradinho *Vigna unguiculata* (L.) Walp] em germinação. **Objetivo**: Nesse trabalho, testamos o medicamento homeopático *Sepia succus*, complementar de *Nat*-m, em plântulas de feijão-fradinho submetidas a estresse salino. **Métodos**: Plântulas de feijão-fradinho desenvolvidas sobre papel de filtro umedecido em placas de Petri foram divididas em 4 grupos: (1) controle em água estéril, (2) em solução de NaCl 50 mM, (3) sementes pré-tratadas com etanol a 90% diluído 1:100 em água e após transferido a solução de NaCl 50mM, e (4) sementes pré-tratadas com *Sep* 200 cH diluídas 1:100 em água e após transferidas a solução de NaCl 50 mM. Num segundo experimento, os grupos foram os mesmos, mas a diluição do etanol a 90% e de *Sep* 200 cH foi 1:1.000 ao invés de 1:00 para reduzir ainda mais o teor de etanol em ambos, medicamento e veículo a fim de minimizar ou abolir o efeito do álcool. Os dados foram analisados através de ANOVA seguida de teste t de Student. **Resultados**: As duas diluições de *Sep* 200 cH, 1:100 e 1:1.000, aumentaram significativamente o crescimento, teor de açúcar, clorofila, proteína e água das plântulas por comparação ao grupo submetido a estresse salino não tratado. O efeito da 1.000ª diluição de *Sep* 200 cH foi mais pronunciado que o

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da 100ª diluição. O veículo etanol a 90% em diluição 1:100 induziu alguns efeitos positivos nas plântulas, mas a diluição 1:1.000 não apresentou esses efeitos. **Conclusões**: *Sep* 200 cH reverteu o efeito de estresse salino nas plântulas de feijão-fradinho, sendo que a 1.000ª diluição foi mais efetiva que a 100ª. O efeito do álcool foi totalmente eliminado com a 1.000ª diluição do etanol a 90%. Portanto, a 1.000ª conservou o efeito do medicamento e eliminou o efeito do veículo.

Palavras-chave: *Sepia succus*, estresse salino, plântulas de feijão-fradinho, crescimento, efeito do etanol, homeopatia.

Sepia 200 cH en dilución 1:1.000 revierte el efecto del estrés salino en plantas de caupí, pero el vehículo etanol al 90% es inefectivo en esa dilución

RESUMEN

Introducción: La salinidad del suelo afecta seriamente la productividad de cultivos en todo el mundo. En estudio reciente, observamos que el medicamento homeopático *Natrum muriaticum* 200 cH mejoró el crecimiento de semillas de caupí *Vigna unguiculata* (L.) Walp] en germinación. **Objetivo**: En este trabajo, testeamos el medicamento homeopático *Sepia succus*, complementario de *Nat-m*, en plantas de caupí sometidas a estrés salino. **Métodos**: Se dividió plantas de caupí desarrolladas sobre papel de filtro humedecido en placas de Petri en 4 grupos: (1) control en agua estéril, (2) en solución de NaCl 50 mM, (3) semillas pre-tratadas con etanol al 90% diluido 1:100 en agua y transferido a solución de NaCl 50mM, y (4) semillas pre-tratadas con *Sep* 200 cH diluidas 1:100 en agua y transferidas a solución de NaCl 50 mM. En un segundo experimento, los grupos fueron los mismos, pero la dilución de etanol al 90% y *Sep* 200 cH fue 1:1.000 en vez de 1:00 para reducir más aun el tenor de etanol en el medicamento y vehículo para minimizar o abolir el efecto del alcohol. Se analizó los datos mediante ANOVA seguida del test t de Student. **Resultados**: Las dos diluciones de *Sep* 200 cH, 1:100 e 1:1.000, aumentaron significativamente el crecimiento, tenor de azúcar, clorofila, proteína y agua de las plantas en comparación conel grupo sometido a estrés salino no tratado. El efecto de la 1.000ª dilución de *Sep* 200 cH fue más pronunciado que el de la 100ª dilución. El vehículo etanol al 90% en dilución 1:100 indujo algunos efectos positivos en las plantas, pero la dilución 1:1.000 no produjo estos efectos. **Conclusiones**: *Sep* 200 cH revirtió el efecto del estrés salino en las plantas de caupí, siendo que la 1.000ª dilución fue más efectiva que la 100ª. El efecto del alcohol fue totalmente eliminado con la 1.000ª dilución del etanol al 90%. Por lo tanto, la 1.000ª conservó el efecto del medicamento y eliminó el efecto del vehículo.

Palabras clave: *Sepia succus*, estrés salino, plantas de caupí, crecimiento, efecto del etanol, homeopatía.

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