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The effect of Leonardite humic substances on the growth and vegetative propagation of saffron (*Crocus sativus* L.)

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Abstract

Saffron (*Crocus sativus* L.) is an autumn-flowering and sterile triploid (2n=3x=24) geophyte species that belong to the Iridaceae family. This triploid plant is propagated by vegetative reproduction through the formation of daughter corms from the mother corm, as the flowers are sterile and fail to produce viable seeds. In fact, many factors hamper the efficient propagation of saffron through this traditional practice and the low multiplication rates of daughter corms under natural conditions reduce productivity, thereby restraining the availability of planting material. Thus, the main objective of this work is to test the effect of humic substances (HS) on saffron vegetative propagation. The main results of trials indicate that HS improves the saffron plant's growth and development, and the intensity of response depends on the concentration used and the mode of application. In fact, the temporary treatment of corms with HS (50 and 100 ppm) improved plant and daughter corms growth but did not affect the number of shoots and corms.

Keywords: Saffron, *ex-vitro*, multiplication, daughter corms production, humic acids, biostimulants

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Effet des substances humiques de la Leonardite sur la croissance et la multiplication végétative du safran (*Crocus sativus* L.)

Résumé

Le safran (*Crocus sativus* L.) est une espèce géophyte triploïde stérile (2n=3x=24) à floraison automnale et appartenant à la famille des Iridacées. Cette plante triploïde se propage par reproduction végétative par la formation de cormes filles à partir des cormes mère, car les fleurs sont stériles et ne produisent pas de graines viables. En fait, de nombreux facteurs entravent la propagation efficace du safran par cette pratique traditionnelle et les faibles taux de multiplication des cormes dans les conditions naturelles réduisent la productivité, limitant ainsi la disponibilité du matériel de plantation. Ainsi, l'objectif principal de ce travail est de tester l'effet des substances humiques (SH) sur la croissance et la multiplication végétative du safran. Les principaux résultats des essais indiquent que les SH améliorent la croissance et le développement du safran, et l'intensité de la réponse dépend de la concentration utilisée et du mode d'application. En fait, le traitement temporaire des cormes avec les SH (50 et 100 ppm) a amélioré la croissance des plantes et des cormes filles mais n'a pas d’effet significatif sur le nombre de pousses et de cormes filles.

Mots-clés : Saffron, e*x-vitro*, multiplication, production de cormes filles, acides humiques, biostimulants

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تأثير المواد الدبالية ليوناردايت على النمو والتكاثر الخضري للزعفران (*Crocus sativus L.*)

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الملخص

يعتبر الزعفران (Crocus sativus L.) نوعا ثلاثي الصبغيات (2n = 3x = 24) وهو عقيم ومزهر في الخريف وهو نوع من النباتات الأرضية (geophyte) التي تنتمي إلى الفصيلة السوسنية (Iridaceae). يتم تكاثر هذا النبات ثلاثي الصيغة الصبغية عن طريق التكاثر الخضري من خلال تكوين بصيلات جديدة من البصيلات الأم القديمة، علما أن الأزهار عقيمة وتفشل في إنتاج بذور قابلة للزراعة. في الواقع، هناك العديد من العوامل التي تعيق الإكثار الفعال للزعفران من خلال الممارسات التقليدية ومعدلات التكاثر المنخفض للبصيلات في ظل الظروف الطبيعية تقلل من الإنتاجية، وبالتالي تقيد توفر بذور قابلة للزراعة. وبالتالي، فإن الهدف الرئيسي من هذا العمل هو اختبار تأثير المواد الدبالية (humiques substances) على التكاثر الخضري للزعفران. تشير النتائج الرئيسية للتجارب إلى أن تطبيق هذه المواد الدبالية تحسن نمو نبات الزعفران وتطوره، وتتباين شدة الاستجابة حسب التركيز المستخدم وطريقة التطبيق. في الواقع، أدت المعالجة المؤقتة للبصيلات بالمواد الدبالية (50 و100 ppm) إلى تحسين نمو النبات والبصيلات ولكنها لم تؤثر على عدد الفروع والبصيلات.

الكلمات المفتاحية: الزعفران، الزراعة الخارجية، التكثير، انتاج البصيلات، الأحماض الدبالية، محفزات حيوية.

Introduction

Saffron (*Crocus sativus* L.), the most expensive spice in the world, is a sterile triploid (2n = 3x = 24) geophyte plant with an underground corm (which belongs to the Iridaceae family) that is generally found in Southwest Asia and the Mediterranean region (Karaoglu et *al.,* 2007; Hajyzadeh et *al.*, 2020; Tahiri et *al*., 2022). Saffron stigmats are widely used as a food additive and for its health-promoting properties due to its secondary metabolites, especially, Crocine, Picrocrocin Safranal (Cardone et *al.*, 2020; Yousefi and Shafaghi, 2020).

Since saffron flowers are sterile and unable to reproduce seeds, the propagation of this species is generally achieved vegetatively through the formation of daughter corms (underground stem) from the mother ones (Tahiri et *al*., 2022). Moreover, under natural field conditions, saffron propagation depends on human labour because the corms need to be manually removed, separated, and replanted. These factors, along with biotic (pathogen infestation) and abiotic stresses, hinder saffron's effective propagation (Parray et *al*., 2012; Menia et *al*., 2018).

Corm size (diameter and weight) is considered a significant factor in determining the saffron's flowering capacity and production of replacement corms (Gresta et *al*., 2008; Kumar et *al*., 2009; Shajari et *al*., 2018). So, creating proper environmental conditions is very important for obtaining corms with larger sizes (Shajari et *al*., 2015; Shajari et *al*., 2018).

Even with strong demand and limited area cultivation, crop output needs to be increased while minimizing environmental impact. This is only possible with the integration of conventional and non-conventional approaches. For this reason, the use of growth regulators or biostimulants has become an important technology to achieve better performance.

Biostimulants are becoming increasingly crucial in the greenhouse and outdoor crop production. They are compounds containing substances and microorganisms that enhance plant nutrient uptake, nutrient efficiency, tolerance to abiotic/biotic stresses, and crop quality (Du Jardin, 2015; Conselvan et *al*., 2017; Parađiković et *al*., 2018). Biostimulants such as humic substances (HS) are used in agriculture and horticulture. In fact, HS are a mixture of small molecules with aromatic rings and aliphatic chains rich in an oxygen-containing functional groups issued from the chemical and microbiological decomposition of organic residues occurring naturally in the environment, particularly in soils, sediments (fossil), natural water and landfills (Tahiri et *al*., 2014). HS are known to influence plant productivity indirectly through modification of soil characteristics and nutrient bioavailability or directly by influencing physiological and metabolic plant processes (Rose et *al*., 2014; Tahiri et *al*., 2014; Tahiri et *al*., 2015; Tahiri et *al*., 2016b; Nardi et *al*., 2017; Conselvan et *al*., 2018).

In order to improve the growth, development and propagation of saffron, this study aims to determine the effect of increasing concentrations and the application method of humic substances on shoot growth and replacement corms multiplication and development.

Materials and methods

Plant material

The plant material dedicated to the *ex-vitro* multiplication trials was collected from a plantation in Tallakht (30.409; -7.787), 22 km from Taliouine, at an altitude of 1630m. The soil has a silty-clay texture. The climate is semi-arid, harsh and very markedly continental. The average annual rainfall is around 200 mm. Only corm with a diameter between 3 and 5 cm and in good sanitary condition are used in these experiments. The tests were carried out at the beginning of November 2019 in an experimental greenhouse (22±3 °C and 50-60% relative humidity) within the Regional Center for Agronomic Research in Agadir (INRA- Morocco).

Chemical characterization of humic substances

The humic substances (HS) used in this study correspond to a commercial formulation extracted from leonardite "Humifirst" and standardized to 12% humic acids and 3% fulvic acids. The chemical characteristics of HS are shown in Table 1. This HS were then analyzed by UV-Visible spectrometer as described in Tahiri et *al*. (2016a). Indeed, the samples were diluted in distilled water and the pH of the solution was adjusted to 6-7. The UV-Vis spectra of the samples were recorded in the wavelength range of 190–800 nm. The E2/E3 and E4/E6 ratios were determined by measuring the absorbance at 254 and 365 nm for E2/E3 ratio and 465 and 665 nm for E4/E6 ratio.

Table 1. Chemical characteristics of Leonardite humic substances (Tahir et *al*., 2016a)

Parameter	Electrical conductivity	Total N	NH_4^+	COD*	Fe	Co	Cr	Cu	Ni	Pb	Zn	As	Cd	Hg
Unit	ms/cm	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
HS*	9.0	80.0	20.0	18770.0	50.00	0.02	0.06	0.09	0.05	0.20	0.11	0.018	0.001	0.0013

***HS**: humic susbtances; **COD**: Dissolved organic carbon

Humic substances Treatment

Two methods of treatments were used in this study:

Temporary treatment

The corms (3< Ø <5 cm) were soaked in different concentrations of HS (0; 50 and 100 ppm) for 6 h and 24 h (the control is soaked in distilled water). The treated corms are then planted in pots (LxlxH: 70x70x150 mm) containing a mixture of sandy soil and peat (1:1). The crops were irrigated with running water once a week until the end of the experiment. 10 corms were used for each treatment and repeated three times.

Continuous treatment

For each treatment, corms (3< Ø <5 cm) were planted directly in pots (LxlxH: 70x70x150 mm) containing a mixture of sandy soil and peat (1:1) at the rate of one corm per pot. The cultures were then irrigated once a week. For treatments, two concentrations (50 and 100 ppm of SH) and a control (distilled water) were applied in the experiment. All treatments were applied every two weeks at the time of irrigation (for 2.5 months). The first treatment was applied at the time of planting. 10 corms were used for each treatment and repeated three times.

Data analysis

Cultures were observed and evaluated regularly every week. The effect of HS was determined when the maximum values were reached (marking the end of the vegetative growth phase) by evaluating aerial part characteristics such as shoots and leaves number and shoots length (cm). Aboveground characteristics such as the daughter corms number, diameter (cm) and weight (g) were recorded at normal corm lifting time when leaves completely turned yellow. The data were then subjected to analysis of variance (ANOVA 1) and Duncan's multi-range test was selected to compare the mean values at the 5% significance level using SPSS software (version 21). Data are presented as mean ± standard error

Results and discussion

Humic substance characterization

The humic substances used in this experiment were first characterized by UV-Vis spectrophotometry before their use for the tests. The UV-Vis spectrum of registered commercial humic substances is presented in Figure 1. In general, the UV-Vis spectrum shows a potential decrease with increasing wavelengths and a strong absorption is observed at wavelengths λ <250 nm, which is characteristic of HS. This strong absorbance could result from the absorption of radiation by the double bonds, particularly the aromatic C=C and ketonic C=O functions of the aromatic chromophores and/or other organic compounds (Chen et *al*., 2002; Tahiri et *al*., 2016a).

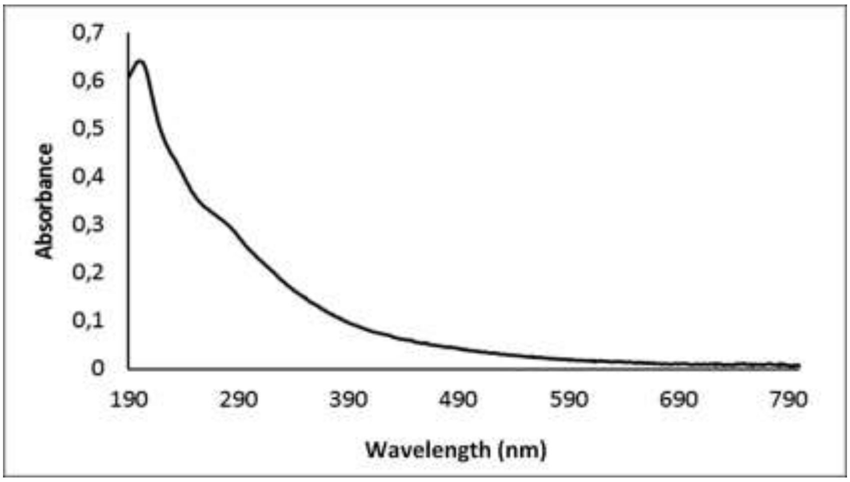

Figure 1. UV-Vis spectrum of Leonardite humic substances

The ratios between absorbance at 254 and 365 nm (E2/E3) and between 465 and 665 nm (E4/E6) were determined (Table 2). These ratios are inversely proportional to the molecular weight and the aromaticity of the molecules but proportional to the contents of O, C and carboxyl groups (COOH) and the total acidity (Chin et *al*., 1994; Uyguner and Bekbolet, 2005; Tahiri et *al*., 2016a). These ratios are often used to characterize HS from different sources. Indeed, a low ratio (<5) indicates a high degree of condensation of the aromatic components, high molecular weight and low acidity. While a high ratio (>5) indicates a greater presence of aliphatic compounds, low molecular weight and high acidity. The results show that the HS used in this work have ratios lower than 5 (2.7 and 2.9), indicating a high degree of condensation of aromatic compounds, a high molecular weight and a low acidity (Table 2). These results show

the dominance of humic acid (HA)-like substances than fulvic acid (FA)-like substances, as reported by Tahiri et *al*., 2016a.

Tableau 2. E2/E3 and E4/E6 ratios of Leonardite SH

Parameter	pH (initial)	pH (adjusted)	E2/E3	E4/E6
	10.2	6-7	2.7	2.9

E2 / E3 (A254 nm /A365nm), E4/E6 (A465 nm /A665 nm)

Effect of humic substances on growth and multiplication of saffron

HS is a promising natural resource to use as an alternative to increasing agricultural production. Thus, in this work, two methods of HS application were tested to evaluate the influence of these substances (as a biostimulant) on the growth and development of saffron.

The effect of increasing concentrations of HS was estimated on the development of shoots and corms of saffron. The results obtained are shown in Figures 2 and 4. These results show that the treatment of saffron corms with HS, by continuous or temporary application, significantly influences the growth and development of both shoots and corms compared to the untreated control.

Continuous application of 50 ppm of HS significantly increases the number of shoots (p=0.001), leaves (p=0.049) and corms (p=0.039) compared to the control, with an average number of 4 shoots, 4 corms and 8 leaves, respectively. However, this treatment significantly reduces the shoots length (35 cm; p=0.05) and the corms weight (1.4 g; p=0.033) compared to the control (38 cm and 2.4 g respectively) (Figures 2 and 4). The application of a high concentration of HS (100 ppm) continuously also showed a significant increase in the number of shoots (5) and corms (5), but significantly reduced the number of leaves to 6, the length of the shoots to 30 cm and the weight of the corms at 1.3g compared to the control (Figures 2 and 4).

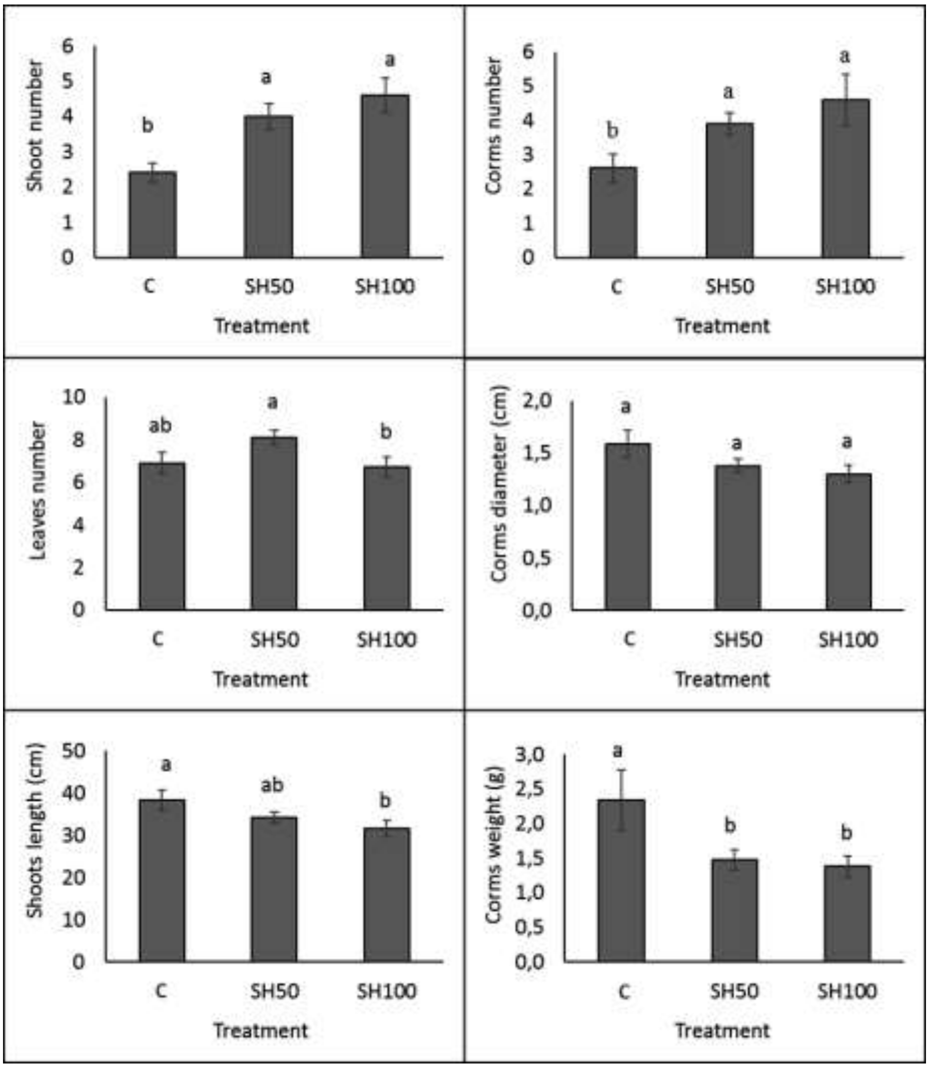

Figure 2. Effects of continuous treatment of saffron with distilled water (C) and HS (50 and 100 ppm) on the growth and development of shoots and corms. Histograms with the same letters are not significantly different at 5% level of Duncan's multi-range test.

The characteristics related to shoots and corms were significantly affected by the temporary application of humic substances (Figures 3 and 4). However, no significant effect ($p>0.05$), compared to the control, was observed for the number of shoots (5) and corms (5) after treatment with 50 ppm and 100 ppm of HS during 6h and 24h, respectively. On the other hand, the treatment of corms with 50 ppm of HS for 24h and 100 ppm for 6h significantly reduced ($p=0.023$) the number of shoots (3.5) and corms (3.5) compared to the control (treated with distilled water (5)).

Regarding the other parameters, a significant increase in the number of leaves ($p=0.000$) and corm diameter ($p=0.003$) was observed after the temporary application of HS, regardless of the concentration and duration of treatment (Figure 3). The maximum leaves number (7 leaves) and corms diameter (Ø = 1.5 cm) were observed after treatment with 50 ppm of HS during 24h and 100 ppm of HS during 6h. In contrast, an increase in shoot length and corm weight of about 20% was observed after treatment with 50 ppm of HS (6h and 24h) and 100 ppm of HS for 6h (Figure 3 and 4).

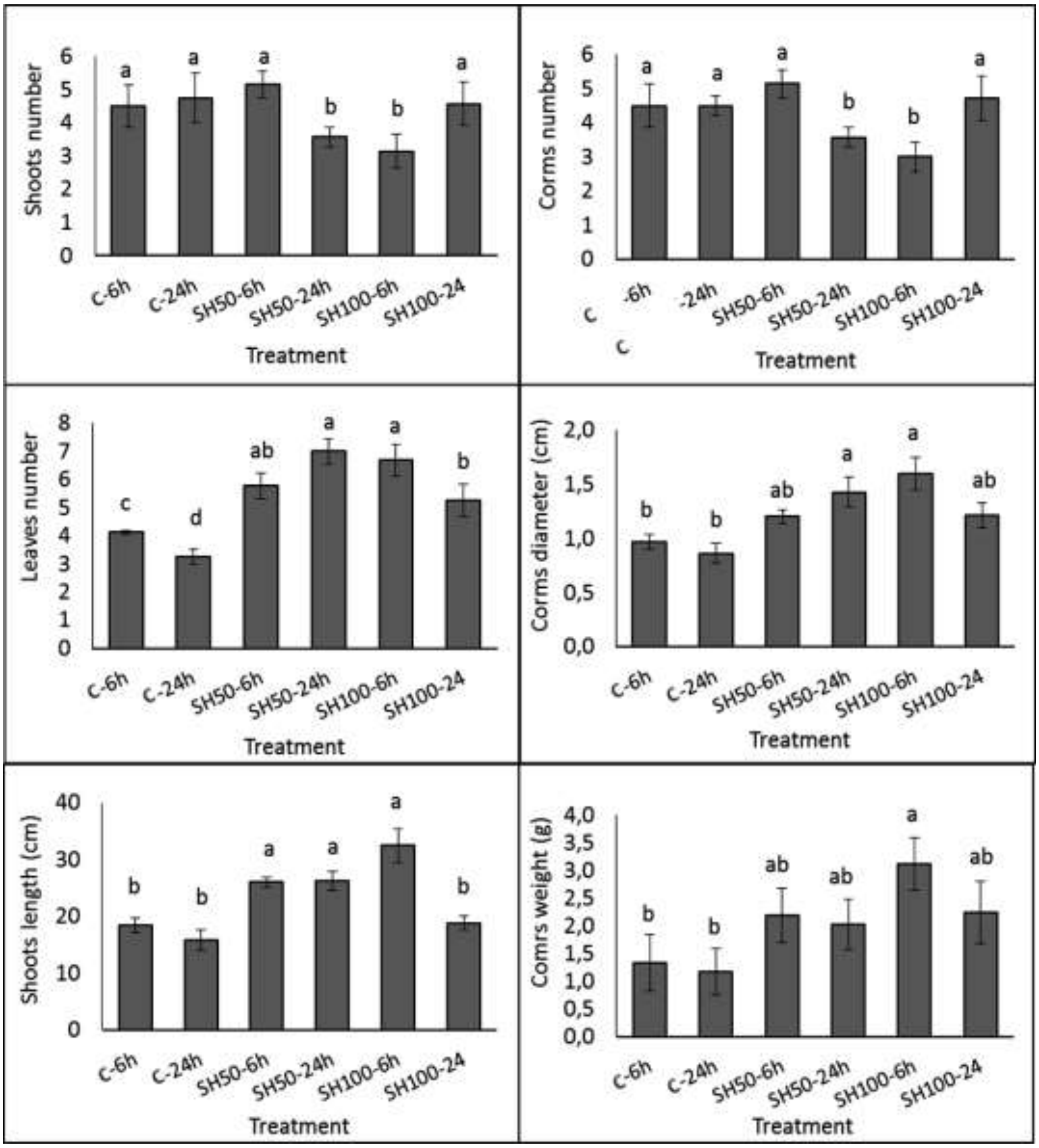

Figure 3. Effects of temporary treatment of saffron corms with distilled water (C) and humic substances (50 and 100 ppm) for 6 and 24 hours on the growth and development of shoots and corms. Histograms with the same letters are not significantly different at 5% level of Duncan's multi-range test.

Humic substances (HS), specifically humic acids (HA) and fulvic acids (FA) of different origins (soil, Leonardite, compost), are known for their beneficial effects on plant development and crop production (Tahiri et *al*., 2014). These heterogeneous complexes can induce morphological, physiological or biochemical variations that manifest themselves in the increase in biomass (Nardi et *al*., 2009; Tahiri et *al*., 2015), germination speed (Chen and Aviad, 1990), cell metabolism (Nardi et *al*., 2009; Trevisan et *al*., 2010; Tahiri et *al*., 2016b), mineral nutrition and therefore crop yield (Verlinden et *al*., 2009). The nature and intensity of the responses vary according to the origin of HS, the nature of the plant species treated and the experimental conditions used (Tahiri et *al*., 2014).

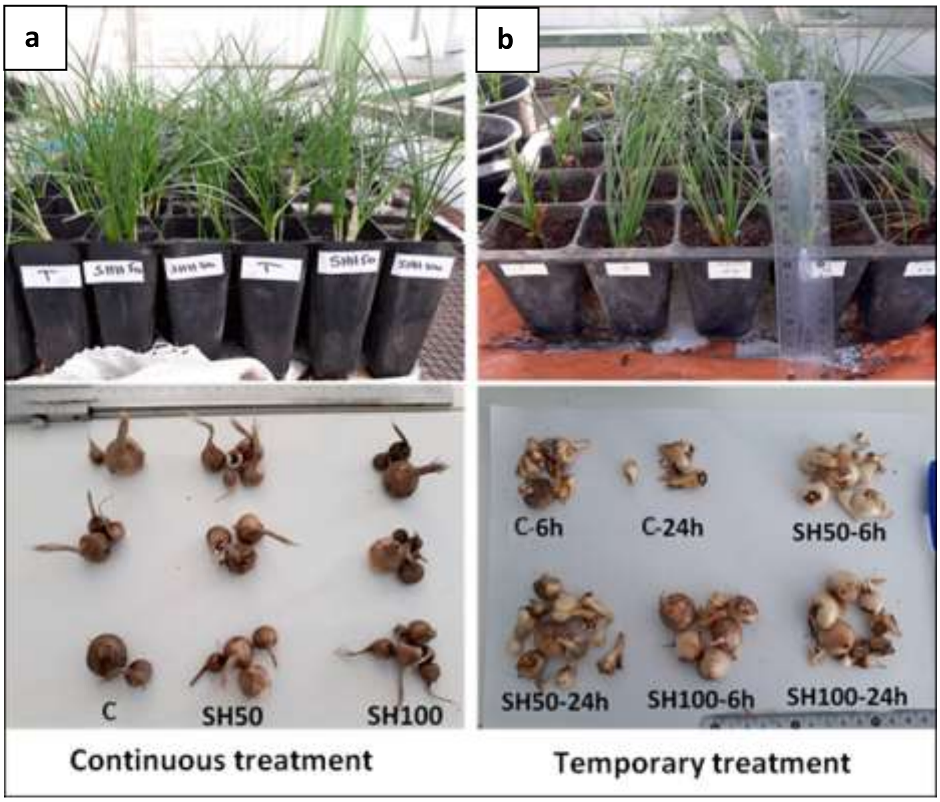

Figure 4. Effects of HS treatment on the growth and development of saffron shoots and corms.**a:** continious treatement with distilled water (C), 50 and 100 ppm HS**. b:** temporary treatment during 6h and 24h with distilled water (C), 50 and 100 ppm HS.

The data obtained in this work show that the effect of humic substances depends on the concentration and application mode. The comparison between the two methods of application of HS shows that the continuous treatment of corms by irrigation (continuous irrigation with an interval of two weeks) made it possible to increase the number of shoots and corms, but it reduced the other parameters. These results partially agree with those of Shajari et *al*. (2018), with minor variations that could be due to the concentration of HS used. These authors showed that humic acid treatment with an irrigation interval of two weeks improved all characteristics of saffron corms. The difference between the results obtained in this work with those of Shajari et *al*. (2018) could be due to the size of the initial mother corms used or the concentration used. While, Koocheki et *al*. (2015), showed that the application of humic acid increases the average weight and diameter of corms by about 33 and 41%, respectively, which contradicts our results.

On the other hand, the temporary treatment influences the growth and development of saffron with a marked improvement in all parameters observed except for the number of shoots and corms (a significant decrease observed in the case of the SH50-6h and SH100-24h treatments).

Conclusion

Faced with the limits of traditional multiplication methods and the growing demand for saffron corms, the selected elite corms will be multiplied under semi-controlled conditions (ex-vitro or in greenhouses). This experiment is one of the few studies on using HS on ex-vitro growth of saffron. The data obtained in this work show that the effect of humic substances depends on the concentration and application mode. Indeed, the soil application method increased the number of shoots and corms, but reduced the other parameters. On the other hand, the temporary treatment made it possible to improve all the parameters observed except for the number of shoots and corms. The choice of the application method will therefore depend on the objective sought. The method of continuous application to the soil is in favour of multiplication, while the temporary treatment is in favour of the growth of the corms. However, other concentrations and other factors (temperature and light) remain to be explored to improve the multiplication and growth of saffron simultaneously.

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