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IN VITRO EVALUATION OF SALT AND DROUGHT STRESS TOLERANCE IN *HYPERICUM CALYCINUM* L.

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Abstract

*Hypericum calycinum L. is a species of the genus Hypericum with pharmaceutical, ornamental, and landscape potential. To assess the salt and drought tolerance stress, in vitro cultures grown on media supplemented with different concentrations of NaCl (0; 10; 30; 50; 100 mM) and PEG 6000 (0; 10; 20; 30; 50 g/l) were used. After six weeks of in vitro culture under salinity and drought stress conditions, the following parameters were evaluated: viability, number of shoots, shoot length, rooting percentage, number of roots, root length, fresh weight, dry weight, water content, stress tolerance index (STI), and McKinney index (MKI). The viability of initial explants was higher under low salt stress (100% under 10 mM NaCl) compared to low drought stress (80% under 10 g/l PEG 6000). Drought stress caused a decrease in shoot height under all concentrations of PEG 6000, while the longest shoots (4.38 ± 0.22 cm) were obtained on the culture medium supplemented with 10 mM NaCl. The rooting percentage was 0% using concentrations of 30, 50, and 100 mM NaCl and 20, 30, and 50 g/l PEG. Our results showed that H. calycinum had a higher sensitivity to drought stress compared to salt stress***.***

Key words*: abiotic stress, drought, in vitro, PEG 6000, salinity.*

INTRODUCTION

Hypericum species are found as wild or cultivated plants on all continents, except Antarctica and their habitus varies from herbaceous plants to trees, belonging to the genus *Hypericum* (Crockett & Robson, 2011; Ma et al., 2021; Danova et al., 2022). Hypericum species are known especially for their medicinal value (Azeez et al., 2017). Some species are also used for ornamental purposes due to their golden yellow flowers, ornamental fruits, and foliage shape (Ji et al., 2017).

Hypericum calycinum L., like other species of this genus, is used for medicinal purposes, especially to eliminate muscle spasms and for the treatment of asthma (Özkan & Mat, 2013). As an evergreen species that blooms from May to August and covers the ground well due to its growth form, it has significant ornamental and landscape potential. *H. calycinum* is used in some areas for landscape design, but its potential is much greater (Yücel et al., 2020). To expand the use of *H. calycinum* in landscape design to other areas, it's important to understand its behavior under drought and saline stress conditions.

In vitro cultures are frequently used to investigate plant responses to various abiotic stresses because they eliminate the influence of environmental factors that are difficult to control in field conditions. Moreover, they enable the rapid evaluation of a large number of species, cultivars, and clones in a small space and without soil impact in experimental fields (Hundare et al., 2022; Vuksanović et al., 2022; Garramone et al., 2023; Wijerathna-Yapa & Hiti-Bandaralage, 2023).

In previous research, saline stress conditions were induced by adding NaCl to culture media (Azzam et al., 2021; Ezzat et al., 2021; Hannachi et al., 2021; Sané et al., 2021; Alenezi et al., 2022; Jalili et al., 2022; Makkar et al., 2022; Asthana et al., 2023; Granata et al.,

2023). To induce drought stress, PEG 6000 was frequently used (Mansinhos et al., 2022; Molnar et al., 2023; Aziz et al., 2023; Beyaz, 2023; Hanif et al., 2023; Mehmandar et al., 2023; Seyed Hajizadeh et al., 2023).

In vitro cultures in media supplemented with different concentrations of NaCl and PEG 6000 have been used to study the response to salt stress and drought stress for many species: *Solanum tuberosum* (Ezzat et al., 2021), *Solanum melongena* (Hannachi et al., 2021), *Solanum lycopersicum* (Sané et al., 2021), *Salvia officinalis* (Alenezi et al., 2022), *Medicago sativa* (Jalili et al., 2022), *Thymus lotocephalus* (Mansinhos et al., 2022), *Vaccinium corymbosum* (Molnar et al., 2023), *Sapindus trifoliatus* (Asthana et al., 2023), *Musa acuminata* (Aziz et al., 2023), *Lotus corniculatus* (Beyaz, 2023), *Ficus carica* (Granata et al., 2023), *Cucumis melo* (Mehmandar et al., 2023), *Rosa damascena* (Seyed Hajizadeh et al., 2023).

This research aimed to evaluate the difference between the response of *H. calycinum* to salinity and drought stress using *in vitro* cultures in media supplemented with different NaCl and PEG 6000 concentrations.

MATERIALS AND METHODS

In vitro treatment of salt and drought stress using NaCl and PEG 6000

To study the effect of salinity and drought on *H. calycinum*, six-week-old *in vitro* cultures grown on Murashige and Skoog, 1962 (MS) culture medium without plant growth regulators (PGRs) were used.

To induce salinity and drought stress, the MS culture medium without PGRs was supplemented with 0; 10; 30; 50; 100 mM NaCl and 0; 10; 20; 30; 50 g/l PEG 6000, respectively. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl before adding the gelling agent. To solidify the medium 5 g/l (w/v) Plant agar was used and the medium was autoclaved for 20 minutes at 121°C. The vessels used consisted of 720 ml (v/v) culture jars (9 cm in diameter and 13.5 cm high) with transparent polypropylene caps.

100 ml (v/v) culture medium was dispensed into each jar and 15 explants were inoculated, each being 1.5-2 cm long.

The *in vitro* cultures were incubated in the growth room at a 16 h photoperiod, 32.4 µmol $m^{-2}s^{-1}$ light intensity (cool white fluorescent light, Philips), and temperature of 23 ± 3°C.

All chemicals were obtained from Duchefa Biochemie B.V, The Netherlands.

Growth Parameters

The growth response to salinity and drought stress was determined after six weeks of in vitro culture conditions, for the following parameters:

- number of shoots - the average number of shoots produced per initial inoculum;
- number of roots - the average number of roots produced per plantlet;
- shoot length (cm) - the average length of shoots produced per initial inoculum;
- root length (cm) - the average length of the roots produced per plantlet;
- mean fresh weight (FW) of shoots per explant (mg): 15 explants for each treatment were weighed immediately after the material was removed from the *in vitro* culture medium;
- mean dry weight (mg): the material was dried for four days at 25°C and re-weighted (DW);
- average water content (WC): based on FW and DW, the WC percentage was calculated using the formula (Mazurek et al., 2021):

$$WC\ (\%) = [(\text{Fresh Weight} - \text{Dry Weight})/\text{Fresh Weight}] * 100$$

Calculation of tolerance indices

Stress Tolerance Index (STI). Based on the collected data, the response to salinity and drought stress was evaluated using the stress tolerance index (STI). The STI for salinity stress was calculated as the ratio between the performance of each parameter at 10, 30, 50, and 100 mM NaCl and the performance traits at 0 mM NaCl. The STI for drought stress was calculated as the ratio between the performance of each parameter at 10, 20, 30, and 50 g/l PEG and the performance of the trait at 0 g/l PEG, according to the following formula (Zaki & Yokoi, 2016):

$$STI = Ts/Tp$$

where:

- *Ts is the trait of genotype under stress treatments;*
- *Tp is the trait of genotype under normal conditions.*

High STI values indicate tolerance to salinity or drought stress.

McKinney index (MKI). Evaluation of the chloroses and/or necroses induced by salinity and drought stress was performed by ranking each shoot into ten classes (Table 1) using a modified McKinney Index (MKI) (Di Cori et al., 2013) according to the following formula:

$$MKI = \Sigma(ni \times i)/N$$

where:

- ni is the number of shoots assigned to the class;
- i is the numeric value of the class;
- N is the total number of examined shoots at each salt and PEG 6000 concentration.

Data are the average value of 30 plants (two jars) for each treatment.

Table 1. The numerical value of the ten classes corresponding to the McKinney index index (MKI)

Class	Symptoms
0	No injury
1	Brownish of basal part of the stem
2	Up to 20% chlorosis
3	From 20 to 50% chlorosis
4	Up to 10% brownish on stem or over 50% chlorosis on whole plant
5	From 10 to 30% brownish on stem
6	Necroses on apical leaves
7	From 30 to 50% necroses on stem
8	Growth reduced or 50% necroses on stem
9	Growth inhibited or necroses on whole stem
10	Necroses on whole plant

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and post hoc testing for the ANOVAs was performed using Tukey's honestly significant difference test (Tukey's test) using a $P<0.05$ significance level to determine the statistically significant differences between the means. Values shown (in text and figures) are means ± SE (standard error).

RESULTS AND DISCUSSIONS

After six weeks of exposure to stress with NaCl and PEG 6000, *H. calycinum* exhibited different responses to the two stress factors (Figure 1).

The viability of initial explants was higher under salinity stress compared to drought stress. Under the lowest concentration of NaCl (10 mM), the viability was 100%, the same as on the culture medium without stress, while under the lowest concentration of PEG (10 g/l) was 80% (Table 2).

The length of the shoots was significantly influenced by the concentration of PEG in the growth medium and decreased significantly under all concentrations (Figure 2). The shoot length decreased 2.8 times under the concentration of 10 g/l PEG, while under the highest concentration of PEG (50 g/l), it decreased 3.8 times compared with the unstressed culture medium.

Table 2. Viability of initial explants under salinity and drought stress

Treatment	Viability of inocula (%)	Rooting percentage
Control	100.00 %	80.00 %
10 mM NaCl	100.00 %	60.00 %
30 mM NaCl	73.33 %	0.00 %
50 mM NaCl	73.33 %	0.00 %
100 mM NaCl	46.67 %	0.00 %
10 g/l PEG	80.00 %	13.33 %
20 g/l PEG	80.00 %	0.00 %
30 g/l PEG	66.67 %	0.00 %
50 g/l PEG	60.00 %	0.00 %

On the other hand, under the lowest NaCl stress (10 mM), the shoots exhibited a longer length (4.38 ± 0.22 cm) compared to the non-stressed culture medium (3.94 ± 0.28 cm), suggesting that *H. calycinum* may be considered a mildly halophytic species.

However, under the highest concentration of NaCl (100 mM), shoot growth was more strongly inhibited (0.39 ± 0.22 cm) compared to the highest concentration of PEG (50 g/l), which resulted in shoots with a length of 1.01 ± 0.07 cm. This result is not unusual, a positive effect of low NaCl concentrations has been reported in other species as well. For example, Zayova et al. (2017) demonstrated that under 50 mM NaCl, plant height and root length were greater compared to the unstressed control under in vitro growth conditions of *Solanum melongena*. Also, in comparison with *H. calycinum*, the length of the shoots of *Viola odorata*, a medicinal and ornamental species, decreased with the increase of both PEG 6000 and NaCl concentrations (Darvishani et al., 2020).

As shown in Figure 3, the number of regenerated shoots was strongly influenced, either positively or negatively, by the two stress factors. Compared to the control, the number of

regenerated shoots decreased under all NaCl concentrations, from 2.22 ± 0.17 (control) to 1.14 ± 0.07 (100 mM NaCl). In contrast, the number of regenerated shoots increased with the increase in PEG concentration. Under the concentration of 50 g/l PEG, the number of regenerated shoots was 3.25, 1.5 times higher than the control. The increase in the proliferation rate in the presence of PEG 6000 was also reported in the case of *Vaccinium. corymbosum* grown *in vitro* under different concentrations of PEG (Molnar et al., 2022).

On the other hand, in strawberries grown in vitro under drought stress, the number of regenerated shoots decreased with the increase in PEG concentration (Clapa & Harta, 2021).

The detrimental effect of these stresses was more pronounced regarding the roots, as indicated in Table 2 and observed in Figure 2. The rooted plantlets were obtained only under low concentrations of NaCl (10 mM) and PEG (10 g/l). When grown on the unstressed culture medium, the plants rooted 80% and under 10 mM NaCl, this percentage decreased to 60%, and under 10 g/l PEG, it plummeted to 13.33%. The number of roots was significantly reduced under 10 g/l PEG (0.22) but remained unaffected under 10 mM NaCl (1.70) compared to the control (1.69). These results highlight the more pronounced detrimental effect of drought stress on root development. The presence of NaCl and PEG in the culture media affected the percentage of rooting, and the number and length of roots in *Stevia rebaudiana* as well (Magangana et al., 2021). The fresh weights and dry weights of shoots decreased under all concentrations of PEG and NaCl, with the most significant reduction observed under 100 mM NaCl (42.60 ± 3.25 mg). The highest shoot fresh weight was recorded in control plants (109.46 ± 13.25 mg) (Figure 4).

Salinity tolerance and drought tolerance are expressed by the stress tolerance index in Table 3. The general trend was a decrease in ITS with increasing salinity and drought levels except for the number of proliferated shoots under PEG concentrations. In this case, the drought tolerance index increased with the increase in the concentration of PEG, confirming the fact that the presence of PEG in the culture medium leads to an increase in the number of proliferated shoots of *H. calycinum*.

To evaluate the degree of chlorosis and necrosis of *H. calycinum* shoots under salinity and drought stress, the McKinney index (MKI) was used (Table 3). No chlorotic leaves or brown shoots were observed in *H. calycinum* plants exposed to 10 mM NaCl, and the MKI value was 0. In contrast, plants exposed to 100 mM NaCl were the most necrotic, with MKI=30.80 (Table 4). MKI shows that plants exposed to drought stress had necrosis under all concentrations of PEG, the most affected being under 50g/l PEG (MKI = 16.80).

Table 3. Stress tolerance index (STI) for *H. calycinum* maintained for four weeks in culture media with different concentrations of NaCl and PEG 6000

Treatment	No of shoots	No of roots	Shoot length (cm)	Roots length (cm)
10 mM NaCl	0.66	1.00	1.11	0.77
30 mM NaCl	0.57	0.00	0.58	0.00
50 mM NaCl	0.61	0.00	0.66	0.00
100 mM NaCl	0.51	0.00	0.10	0.00
10 g/l PEG	1.07	0.13	0.35	0.24
20 g/l PEG	1.41	0.00	0.30	0.00
30 g/l PEG	1.35	0.00	0.28	0.00
50 g/l PEG	1.46	0.00	0.26	0.00

Table 4. McKinney index (MKI) for *H. calycinum* maintained for four weeks in culture media with different concentrations of NaCl and PEG 6000

Treatment	MKI
Control	0.00
10 mM NaCl	0.00
30 mM NaCl	0.83
50 mM NaCl	3.33
100 mM NaCl	30.80
10 g/l PEG	0.20
20 g/l PEG	0.83
30 g/l PEG	8.67
50 g/l PEG	16.80

Salinity and drought stress induced in this study by supplementing the culture media with different concentrations of NaCl and PEG showed that *H. calycinum* has a higher sensitivity to drought stress compared to salinity stress (Figure 5).

Our results showed that high salinity and drought caused a reduction in the growth of shoots and roots of *H. calycinum* grown in vitro and confirmed that the strategy of stressed plants is to slow down their growth (Hossain et al., 2007).

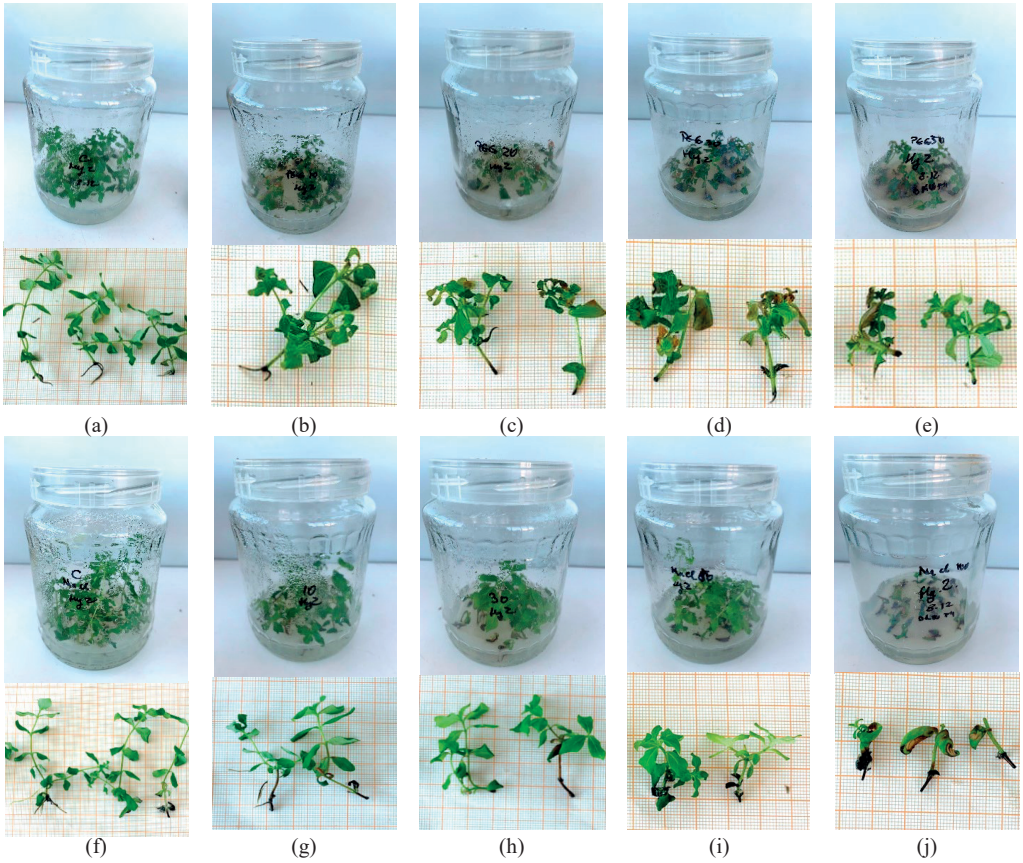

Figure 1. Effect of different concentrations of PEG 6000 and NaCl on in vitro culture of *H. calycinum*: control (a, f), 10 g/l PEG 6000 (b), 20 g/l PEG 6000 (c), 30 g/l PEG 6000 (d), 50 g/l PEG 6000 (e), 10 mM NaCl (g), 30 mM NaCl (h), 50 mM NaCl (i) and 100 mM NaCl (j)

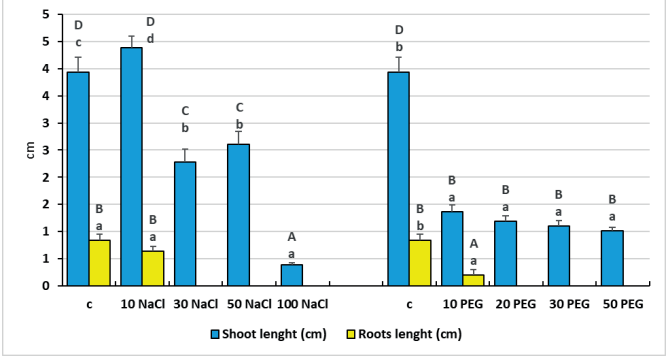

Figure 2. The length of shoots and roots of *H. calycinum* under salinity stress (0, 10, 30, 50, and 100 mM NaCl) and drought stress (0, 10, 20, 30, and 50 g/l PEG 6000). Different lowercase letters indicate significant differences among the same treatments and different capital letters indicate significant differences among all treatments according to Tukey's HSD test ($P \leq 0.05$). Error bars indicate mean ± SE

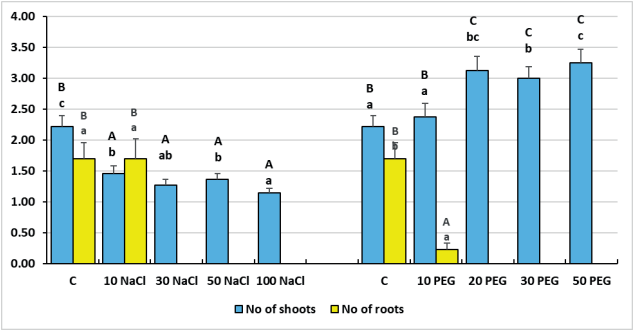

Figure 3. Number of shoots and number of roots per initial inoculum of *H. calycinum*. For inducing the salinity stress in culture media were added 0, 10, 30, 50, and 100 mM NaCl and for inducing drought stress were added 0, 10, 20, 30, and 50 g/l PEG 6000. Different lowercase letters indicate significant differences among the same treatments and different capital letters indicate significant differences among all treatments according to Tukey's HSD test ($P \leq 0.05$). Error bars indicate mean ± SE

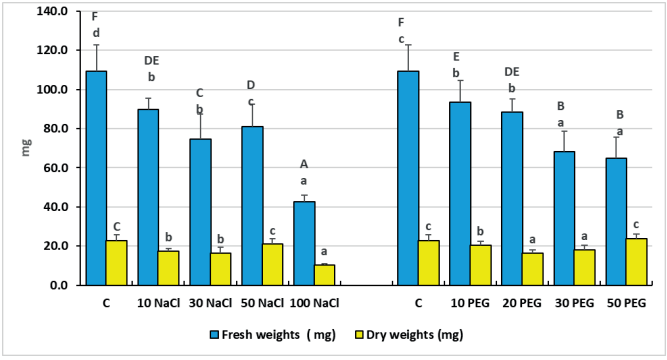

Figure 4. Fresh weights (mg) and dry weights (mg) of the shoots of *H. calycinum* under salinity stress (0, 10, 30, 50, and 100 mM NaCl) and drought stress (0, 10, 20, 30, and 50 g/l PEG 6000). Different lowercase letters indicate significant differences among the same treatments and different capital letters indicate significant differences among all treatments according to Tukey's HSD test ($P \leq 0.05$). Error bars indicate mean ± SE

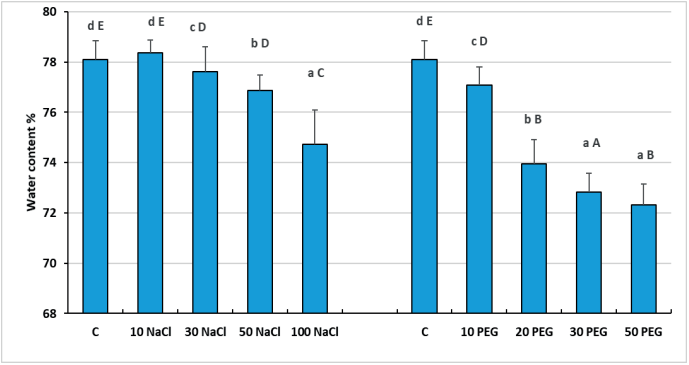

Figure 5. Water content of the shoots of *H. calycinum* under salinity stress (0, 10, 30, 50, and 100 mM NaCl) and drought stress (0, 10, 20, 30, and 50 g/l PEG 6000). Different lowercase letters indicate significant differences among the same treatments and different capital letters indicate significant differences among all treatments according to Tukey's HSD test ($P \leq 0.05$). Error bars indicate mean ± SE

CONCLUSIONS

In conclusion, our results showed that *H. calycinum* exhibits greater sensitivity to drought stress compared to salt stress. Specifically, the height of the shoots was higher at low salt concentrations (10 mM NaCl) compared to those obtained in the absence of stress factors. This suggests that *H. calycinum* may be suitable for landscaping in areas with lands with a low degree of salinity. To validate this finding, field research should be extended.

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