CHLOROPHYLL CONTENT AND STOMATA MORPHOMETRIC FEATURES OF ANETHUM GRAVEOLENS L. IN A CONTROLLED EXPERIMENT WITH DIFFERENT SALINE LEVELS

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Abstract

The intensive use for different purposes of dill requires split and even short-term studies that are desirable in purpose to highlight the threatening thresholds of salinity doses during vegetation growth season. The aim of the study was to assess the dill growth according to BBCH scale, the leaves' chlorophyll content and stomata number, and morphological characteristics during growth and development under salinity stress. The experiment was set under controlled conditions at $20\pm2^{\circ}$ C T, and 40% H under full light in the growth chamber. Two different salinity levels of 50 mM, 100 mM NaCl and a control were tested in 3 treatments for 5 plants in 6 repetitions. Dill germination rate registered the higher value at 50 mM NaCl compared to the control treatment. At the end of the 10 BBCH germination stage, the lowest NaCl dose determined 100% of germination capacity. The higher stomata density was in the treatment with 50 mM NaCl, with 46% more compared to the control. The chlorophyll content of dill decreased with the increasing salinity levels. Dill is not negatively influenced by the two salinity doses tested.

Key words: BBCH scale, dill, germination, physiological parameters, SPAD units.

INTRODUCTION

Medicinal and aromatic plants (MAPs) are very important for the economy, being highly used for various purposes like alternative medicine, the cosmetic industry, and most important in global alimentation due to their aromatic scent and also for their antioxidant and relaxing properties (Stoian et al., 2022).

An annual plant, dill (Anethum graveolens L.) is part of the Apiaceae family. Native from Central and Southeast Asia, but also found in the Mediterranean area of Europe. It is recognized for its qualities as an aromatic and medicinal plant (Kaur et al., 2021). Dill is used to adding flavor to salads, soups, and dishes, but also for its therapeutic qualities, especially against digestive problems. Dill seed essential oil contains carvone, limonene, and alphaphellandrene (Wall et al., 1986). Along with the other chemical components of the plant, they ensure effects such as antimicrobial, antiinflammatory (Abdel-Aziz et al., 2016), analgesic, and calming problems of the gastrointestinal tract and in the female reproductive system conditions (Al-Snafi et al.,

2014). Together with other aromatic plants, dill could be susceptible to different abiotic stress. One of the most important stressors is considerend salinity stress which intensifies in large areas globally in the current context of climate change.

Salinity is an abiotic stress that represents a threat to agriculture, inhibiting and prohibiting the plant's physiological mechanisms (Yadav et al., 2020). Saline stress restrains the proper nutrition of the plant, a fact that ultimately leads to the defective production of secondary metabolites, the main components of the MAPs essential oils (Ghassemi-Golezani et al., 2022). Essential oils represent a standard in the assessment of this group of plants quality (Li et al., 2020). Even though salinity can delay or limit the development of aromatic plants, there are also species of medicinal plants that manage to grow in an environment with low salinity, such as Achillea millefolium and Matricaria chamomilla (Máthé et al., 2015). Although MAPs are intensively used both in food and in alternative medicine, being the first treatments used by humans (Marshall, 2011), in Romania. their vegetable agricultural

production decreased from 20.459 tons in 1990 to 3.313 in 2021 (INSSE, 2021). In the climate change context, drought, and intensive use of irrigation, soil salinization is a serious consequence that has a various series of effects on the growth and development of plants (Okur et al., 2020). The establishment of osmotic stress caused by water deficit and nutritional imbalance are followed bv adaptation mechanisms of plants, including the accumulation of proline (Heuer, 2010). proteins, and soluble carbohydrates (Pirzad, et al., 2011). It can also lead to a decrease in the level of phenolic content, therefore reducing the antioxidant potential of plants. These biochemical imbalances lead to a reduction in the intensity of photosynthesis, the closing of the stomata, and a decrease in the seed germination rate (Chaves et al., 2009). Regarding medicinal plants, the most important physiological process is precisely germination, a process initiated by germplasm activation (Moldovan et al., 2022) and is strongly influenced by the soil salinity. Plants have different degrees of salinity tolerance, and aromatic and medicinal plants are largely resistant to decreased levels of salinity. The concern is the influence of saline stress on the level and composition of secondary metabolites biosynthesized. There is a fine line between benefits and toxicity, reason why it is important to correctly classify plants according to the salt resistance degree and to identify the salt maximum dose that can redefine medicinal plant into a toxic one. Some studies mention Melissa officinalis and Cuminum cyminum as sensitive to salinity by decreasing the germination rate and percentage (Younesi and Moradi, 2014). According to Mathe, Achillea millefolium and Matricaria chamomilla are plants resistant to low levels of salinity (Máthé et al., 2015). On the contrary, Said-Al Ahl places Ocimum basilicum and Matricaria chamomilla in the category of plants sensitive to salinity, the stress-inducing the defective mobilization of reserve substances and suspending cell division (Said-Al Ahl and Omer, 2011). Ocimum majorana records in the presence of 100 mM NaCl a decreased essential oils content (Li et al., 2020). Coriandrum sativum showed a decrease only in the case of very high values of NaCl, and an

lemongrass *Cymbopogon citrates* (Aishwath and Lal, 2016). Previous studies on dill *Anethum graveolens* salt tolerance and how its physiological processes are affected are few, the plant was included in the group of plants with resistance to decreased levels of salt (Aishwath and Lal, 2016). On the contrary, Soliman and Abou-Ellail reported that dill germination is not

impressive increase in essential oils was found

at 25-50 mM NaCl (Li et al., 2020). Even

though salinity can delay or limit the

development of aromatic plants, there are also

species of medicinal plants that manage to

grow in an environment with salinity, such as

winterianus

and

Cymbopogon

citronella

Ellail reported that dill germination is not influenced by salinity, but during the early growth period, the plant is very sensitive to the saline stress (Soliman and Abou, 2016). Reason of which, the aim of the study was set to test de dill growth according to BBCH scale. Hereby were assessed the leaves' chlorophyll content and stomata number, together with morphological characteristics during growth and development under salinity stress.

MATERIALS AND METHODS

The experiment conducted under was controlled conditions. A growth chamber was used with the following constant properties: intensive light from 20 of 18 W neon lights, humidity of 40% and 20±2°C temperature. The tested treatments have consisted of a number of three variants respectively 50 mM NaCl, 100 mM NaCl, and 0 mM NaCl as control, in six replications for 28 days. Saline doses in 50 ml water were applied every three days during watering time. Dill Anethum graveolens L. seeds were purchased from Dr. Soil GmbH Germany. Previously to the experiment, the seeds were sterilized using a 50% ethanol solution for 15 minutes in circular moves, so that the seeds flote above solution and then were rinsed for three times with 100 ml distilled water (Lindsey et al., 2017). All the viable seeds were placed in 25 g soil. The soil substrate had a pH between 5-6.5 and a N content<1.9% in 150-250 mg/l; P(P2O5) <0.3% in 100-200 mg/l; K(K₂O) <0.7% in 200-300 mg/l from AGRO CS, Romania. During the experimental time, the number of germinated

seeds was daily assessed together with the seed development stage on BBCH scale. This scale represents the newest standardized growth and development monograph for plants (Meier, 2018). Here are comprised principal and secondary growth stages from the vegetation period of a plant.

According to Sedghi et al., germination percentage (GP) was calculated as the proportion of viable seeds in a given population, and is determined by dividing the number of seeds (n) that have successfully germinated by the total number of tested seeds (n T) (Sedghi et al., 2010).

$\text{GP }\% = \boldsymbol{n}/\boldsymbol{n}\,\boldsymbol{T}\times 100$

The germination rate **GR** was established for each treatment in the day where around half of tested seeds succeed to germinate and it was calculated as a percentage (Liopa-Tsakalidi et al., 2011).

Chlorophyll content was measured using the MC-100 S/N Apogee Instruments chlorophyll meter, in SPAD units for each plant at the end of the experiment. This is a non-destructive method and gives accurate measurement of instant values. Before each read, the chlorophyll meter was calibrated and the values represent absolute μ mol m⁻¹.

Stomata imprints were collected at the end of the experiment. The leaf adaxial surface was covered with red nail varnish then after preliminary drying, sticky tape was used to peel the leaf imprint and placed under a microscope glass slide. The slide prints were assessed under the microscope Olympus CX43 and the images were captured with camera model PROMICAM PRO4K of 12 MP.

The database was analyzed with RStudio software, Anova and Fisher LSD were performed at p<0.05 for dill leaves chlorophyll content under salinity treatments in SPAD units, the parameter chlorophyll content was presented as average data \pm standard error (S.E.).

Percentage values were projected for germination rate and for BBCH secondary stages distribution also on all performed assessments. Respectively for the other parameters like stomata aperture and density were presented average values and standard deviation (S.D.) on the figures.

RESULTS AND DISCUSSIONS

The most favorable treatment for dill germination was at 50 mM NaCl. In the first germination assessment (D1), after two days' germination capacity values of 100% were recorded in two of the sixth repetitions (Figure 1). On the second assessment of the experiment (D2 after 4 days), maximum germination capacity was recorded in all variants with 50 mM NaCl tested.



Figure 1. Dill germination rate (GR) and capacity (GP) in percentages under salinity stress (Dill 0-control, Dill 50-50 mM NaCl, Dill 100-100 mM NaCl)

On average, dill seeds from the 100 mM NaCl treatment showed a very similar trend to the control.

The maximum germination capacity of dill seeds of the control variant was reached after eight days compared to 50 mM NaCl variant which registered the maximum germination capacity on the fourth day. The time to reach the maximum germination percentage with 50 mM NaCl Dill 50 treatment was 4 days, reduced by 3 days compared to the same dose applied in the Liopa-Tsakalidi et al (2011) study.

Dill seeds from the 100 mM NaCl treatment had a germination capacity of 96% on the sixth day of the experiment. This percentage did not change until the end of the experiment, probably due to the fact that for 4% of the dill seeds, the saline dose showed a slightly toxic, germination-inhibiting effect.

In the case of the control and the 100 mM NaCl treatment, four out of six replicates were represented by seeds with 100% germination capacity. The germination percentage is not affected by the reduced saline dose. The high NaCl dose (100 mM Dill 100) can affect PG, which was also proven by previous studies by Ravender and Kundu (2000). According to Liopa-Tsakalidi et al (2011) study, the

application of a dose five times higher than the highest dose in our study (100 mM NaCl) inhibited the germination process of 11 MAPs species, of which, one was the dill. Similar to a study conducted in 2011 (Liopa-Tsakalidi et al., 2011), the dill seeds germination in our study was only slightly affected by a concentration higher than 50 mM NaCl, i.e. 100 mM NaCl. Similar to the Liopa-Tsakalidi et al. studies (2011), the germination percentage at the most concentrated dose tested in our study was not significantly affected. The 2011) studies (Liopa-Tsakalidi et al.. mentioned above onlv report that concentrations from 500 mM NaCl affect germination.

The seeds germination rate subjected to salinity stress differed according different tested dose. At the 50 mM NaCl treatment, the germination rate for dill was with 12% higher than in the control variant. For seeds subjected to 100 mM NaCl, this process was reduced three times with a difference of 4% higher compared with the control. Contrary to what was reported in a 2000 study (El-Darier and Youssef, 2000) on the germination rate of cress seeds, the highest GR value in our study was recorded at the double saline dose respectively at 100 mM NaCl Dill 100.

The resistance of the germination process to salinity varies depending on the plant and its variety, but also on the salt stress intensity. Under high salinity conditions, the germination percentage and rate can be enhanced by applying GA_3 which alleviates the harmful effects of sodium accumulation (Sedghi et al, 2010).

The most higher dill seed percentage in BBCH 09 - emergence - cotyledons breaks through soil surface, at the first assessment (D1) was in the treatment with 50 mM NaCl (Dill 50) (Figure 2). This percentage was higher that the control with 13% and the most lower seeds percent of 23% in this secondary stage was in the treatment with 100 mM NaCl (Dill 100) compared with the control.

At the second assessment (D2) after 4 days, the seeds followed the same trend as in the previous assessment only regarding the maximum percentage value of the Dill50 treatment, the seeds reached BBCH 09 in the same percentage of 90% in the control and Dill100 treatments.

At the third assessment (D3) after 6 days it was observed an accelerated development of secondary stage at the treatment Dill 100 completely into BBCH 10 represented by completely unfolded cotyledons with 3% higher value compared with the previous assessment. Dill 50 was represented by seeds in BBCH 10 in the most lower percentage with 80% compared with the control. After 8 days (D4), the Dill 50 seeds in the following BBCH secondary stage (BBCH 11 with first true leaf unfolded) represented 24% and had a 7% increase compared with the control also registered a decrease of 21% in Dill 100. In the next assessment (D5), the seed percentage in BBCH 11 in Dill 50 doubled. Therefore, compared to the control variant this value was with 30% higher and compared to Dill 100 was with 37% higher. After this assessment (D5) dill seeds from Dill 50 treatment develop further in secondary stages and register the most higher percentages. The percentage difference (D6-D5) of Dill 50 compared with control had decreased to 19% by 11% and compared to Dill 100 in D5 the difference from D6 increased to 53% by 16%. Approximately the same difference it was observed on D7 assessment between Dill 50 and control variant. The difference between Dill 50 and Dill 100 was similar with the D5 percentage of 37%. Representative secondary stages at D8 for Dill 50 and Dill 100 treatments was BBCH 12 (with 2 true leaves unfolded) and BBCH 11 (with first true leaf unfolded). The control variant has an extra 5% of dill seeds in BBCH 10, an inferior developmental secondary stage. Therefore, D8 it can be considered an important threshold to differentiate the speed of seedlings leaf development. Dill seedling growth length. Dill seedlings growth and development were affected by salt stress at concentrations higher than 50 mM (Liopa-Tsakalidi et al., 2011). More than half (67%) of germinated seeds from Dill 50 were in BBCH 12 with a difference of 21% higher compared to the control and 40% higher than Dill 100. Only after 16 days (D8), dill seeds percentage in BBCH 11 from Dill 100 was the highest (66%) with 20% higher than the control and with 36% higher in comparison with Dill 50.

At the assessment D9, after 18 days, only in Dill50 were seen seeds in BBCH 13 with already 3 true leaves unfolded in a relatively low percentage of 3%. This last secondary stage was observed to the other two treatments only after 6 days after three assessments (D12). In comparison with control treatment at D9, in Dill 50 were with 23% higher dill seedlings in BBCH 12 and with 14% lower in Dill 100. Secondary stage BBCH 11 was represented by the most higher percentage (53%) in Dill 100 with of difference of 10% from the control and 36% compared with Dill 50. All the tested treatments can be further classified based on development under BBCH secondary stages until the end of the experiment. The most active seeds and leaves of fast growing seedlings were observed in Dill 50, followed by the control treatment and Dill 100. In D12

assessment, both the control and Dill50 treatment had a seedlings frequency percentage of leaves development in BBCH 12 and BBC3 13 similar both on the secondary stages and also between the two treatments tested. So, the control variant overcomes the percentages from 50 mM NaCl in D12 compared with the previous assessments. At the end of the experiment, the seedlings in BBCH 13 under Dill50 registered 25% difference compared with the control and 39% difference compared with Dill 100. This percentage trend seen in D13 was similar with assessment D5. After evaluating BBCH distribution in secondary stages for each day, it is obvious that the level of quantifying each response at salinity exposure it has a high degree of difficulty (Poljakoff-Mayber and Gale, 1975).



Figure 2. Percentage of germinated dill seeds and seedlings development for each BBCH secondary stage in all assessments (D1, D2...D13) for each treatment (Dill 0-control, Dill 50-50 mM NaCl, Dill 100-100 mM NaCl)

The control variant seedlings recorded the highest chlorophyll content, 44.8 ± 6.0 within a range between 36.9 and 56.5 SPAD units (Figure 3). The low salinity concentration Dill50 seedlings had slightly low value of SPAD units with around 5% compared to the control variant.

The lowest value of chlorophyll content was reported in the high concentration of 100 Mm NaCl around 35.5 ± 0.8 (SE), which registered a

decrease of 16.5% compared to the 50 mM NaCl variant and with 21% compared to control.

Contrary to Shekari et al study (Shekari et al., 2017) reporting that under saline conditions at a conductivity of 10ds/m, equivalent to a concentration of about 100 mM (Dheeravathu et al., 2018), respectively the high concentration in our study, chlorophyll levels did not show significant decreases between 0–

50 mM NaCl treatments. In the case of our study, although the tendency of chlorophyll content is to decrease with increasing salt concentrations, the decrease in chlorophyll level between treatments is not significant, contrary to the studies of Hassanpouraghdam et al. (2022). Decreased chlorophyll content may be associated with elevated levels of chlorophyllase enzyme activity (NikpourRashidabad et al., 2016). Other causes of low chlorophyll levels are due to the acceleration of the degradation process of photosynthesis and the decrease of biosynthesis of the essential substance in the photosynthesis process, both processes being consequences of the installation of oxidative stress (Shekari et al., 2017).



Figure 3. Dill leaves chlorophyll content under salinity treatments in SPAD units (Dill0-control, Dill50-50mM NaCl, Dill100-100mM NaCl). Fisher LSD test, different letters represent significant differences at p<0.05.



Figure 4. Dill stomata morphometric representative features (a) Dill 0 at 10x-200 μm scale;
(b) Dill 0 at 20x-100 μm scale; (c) Dill 0 at 40x-50 μm scale; (d) Dill 50 at 10x; (e) Dill 50 at 20x;
(f) Dill 50 at 40x; (g) Dill 100 at 10x; (h) Dill 100 at 20x; (i) Dill 100 at 40x

In the control variant at 10x magnifying (Figure 4, a), the stomata are positioned interspersed along the longitudinal profile of the dill leaf imprint. Their distribution can be characterized as uniform over the leaf surface. The 20-fold magnification image shows details of 5 sides of the annex cells distributed on both sides of the main venation (Figure 4, b). On average, the aperture width is around 25 um. the cell walls are rounded at the annex cells and have a length of around 50 µm (Figure 4, c). Under the second treatment (Dill 50), stomata have slightly larger aperture than the control (Figure 4, d). Cell walls were altered, perhaps due to salt stress presence, at the lowest dose tested. The cell shape could be characterized by curved lines with 6-9 smooth peaks (Figure 4, e). The doubling of stomatal cells is a counteracting mechanism of saline dose (Figure 4, f). Cells are lax and distorted, placed discontinuously or with large spaces between them compared to the control. Stomata have thickened walls and apertures up to twice as large as the control (Figure 4, g, h, i). The irregular shape of the cells is due to the accumulation of potassium ions in the guard cells. Increased osmotic pressure was proven to be a consequence of ion imbalance installed after salt stress (Wilmer and Fricker, 1996).

In the middle leaf area (Dill 0 M), the average value was the highest 4 ± 1.1 , with a maximum of 5 µm stomatal pore aperture or opening between guard cells (Figure 5). On the base of the leaf (Dill 0 B), the average value was 3 ± 0.6 with a minimum of 2 µm. The average adaxial stomata pore aperture on the control (Dill 0 T) leaf tip area was 2 ± 0.6 within the range of 1-3 µm.





Dill0 B-stomatal pore aperture on leaf base)

For Dill50 the middle leaf area shows the same trend ass Dill 0 (Figure 6). On the middle leaf area (Dill 50 M), the average value was 4.2 \pm 0.7, with a maximum of 5 µm stomatal pore aperture. The average stomata pore opening on 50 mM NaCl treatment (Dill 50 T) leaf tip area was 2.8±0.7 within the range of 2– 4 μ m. On the base of the leaf (Dill 50 B) the average value was 4 ± 1.1 with a minimum of 2 µm.



Figure 6. Dill leaves adaxial stomatal pore aperture on 50 mM NaCl treatment (Dill 50 T-pore aperture on leaf tip area, Dill 50 M-stomatal pore aperture on middle leaf area; Dill 50 B-stomatal pore aperture on leaf base)

The adaxial stomata pore aperture on 100 mM NaCl treatment (Dill 100 T) leaf tip area was 4 ± 0.8 within the range of 3-5 µm (Figure 7), double that in the control treatment. In the middle leaf area (Dill 100 M), the average value was 1.8 \pm 0.4, with a maximum of 2 μ m stomatal pore aperture, twice as low as the Dill 0 M. On the base of the leaf (Dill 100 B), the average value was 1.8 ± 0.7 with a minimum of 1 µm.



Figure 7. Dill leaves adaxial stomatal pore aperture on 100 mM NaCl treatment (Dill100 T-pore aperture on leaf tip area, Dill100 M-stomatal pore aperture on middle leaf area; Dill100 B-stomatal pore aperture on leaf base)

Compared to the control, the stomatal pore aperture on the tip of the leaf in Dill 50_T is with 40% higher than in Dill 0_T (Figure 5). On Dill 100_T, in the tip of the leaf, the adaxial stomata opening is 100% higher than the control (Figure 6). The average value in Dill 50_M was similar to Dill 0_M (Figure 6). In Dill 100_M the stomatal aperture had decreased by 55% compared to the Dill 0_M value (Figure 7). In Dill50_B, the average stomata pore opening was 33% higher than the control Dill 0_B (Figure 6). Dill 100_B registered a decreased average value of the pore opening with 40% compared to the control (Figure 7).

Another similar study, on barley, reported a significant decrease of stomatal pore aperture with the increasing concentration of the saline solution at four treatments (0, 50, 100, 150 mM NaCl) (Hassan et al., 2021). Contrary to this study, our experiment reported a higher average stomatal pore aperture in the 50 mM NaCl variant Dill50 compared to the control variant Dillo. The 100 mM NaCl Dill 100 variant recorded lower average values of stomatal pore aperture compared to the 0 mM NaCl Dill 0 control variant and the 50 mM NaCl Dill 50 variant, similar to the trend reported in Hassan et al., 2021 study. The inhomogeneous appearance of the stomatal pore aperture may be a consequence of sudden changes in environmental factors and is leading changes in the functioning of the to transpiration and photosynthesis processes (Wilmer and Fricker, 1996).

The most highest density of stomata per leaf area in cm^2 , was observed in the Dill 50 treatments (Figure 8).



Figure 8. Dill leaves stomatal density (average stomata per total leaf area in number±S.D./cm²) under salinity (Dill 0-control, Dill 50-50 mM NaCl, Dill 100-100 mM NaCl)

Compared with the control variant Dill 0, this value is with a number of 135 stomata more. The higher salinity dose from Dill 100 had a negative effect of the stromata number, therefore this was lower with 29 compared with the control.

The largest leaf area was recorded for the Dill50 version, i.e., 730 mm². The smallest leaf area was recorded for the control variant Dill 0 amounting to 400 mm². With a similar value, the leaf area of Dill 100 represents an area of 450 mm^2 .

The highest stomata density value recorded for Dill 50 was 429 ± 134 (S.D.) stomata/leaf area and was represented by a 46% increase compared to the stomata density value of Dill 0 (294±81 stomata/leaf area). The lowest stomata density at leaf area level was recorded for Dill 100, i.e. 265±23 stomata/leaf area with 10% lower compared to control - Dill 0.



Figure 9. Dill under salinity stress experiment in growth chamber, leaves and leaves imprints for assessment of stomata features

On barley, the stomatal density increases significantly with increasing salt concentration (Hassan et al., 2021). A trend similar to that reported in this study is manifested by dill at the 50 mM NaCl Dill 50 variant. In contrast, the 100 mM NaCl Dill 100 variant recorded a decrease in stomatal density compared to the 0 mM NaCl Dill 0 control and to the 50 mM NaCl Dill 50 variant, contrary to the previously mentioned study.

CONCLUSIONS

The fastest and highest germination capacity value was recorded for the 50 mM NaCl variant on day 4.

The highest germination rate value was also recorded on day 4 but at the 100 mM NaCl variant.

The 0 mM NaCl control variant recorded the highest chlorophyll content expressed in SPAD units. However, chlorophyll levels did not vary significantly with increasing salt concentration. At 50 mM NaCl concentration, the Dill 50 variant showed high values of germination percentage, stomatal pore aperture, leaf area and stomatal density, indicating a high tolerance of the plant to this level of salt stress.

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