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RESEARCH ARTICLE

Influence of extrinsic factors and salt stress on the *in vitro* germination and development of Moroccan *Cannabis sativa* L. varieties as potential medicinal plants

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Abstract

The optimization of *Cannabis sativa* L. germination is vital for achieving controlled and uniform cultivation, especially given its growing significance as a medicinal plant rich in bioactive compounds. Since germination strongly impacts subsequent agronomic performance, it requires specific environmental conditions. This study assessed several treatments to improve the *in vitro* germination of two Moroccan varieties, Beldia and Khardala, including gibberellic acid, hydrogen peroxide, temperature, photoperiod exposure, total darkness, mechanical scarification and salt stress induced by increasing concentrations of NaCl (0, 42.8, 85.5, 171.1, 256.6 and 513.3 mM). Each treatment was performed on a large number of seeds per variety and results were analysed using two-way ANOVA followed by Duncans' test with $P < 0.05$. Results revealed that photoperiod exposure moderately stimulated germination (50 %), while hydrogen peroxide (1.5 %) increased it to 77.7 % by reducing the level of abscisic acid, which is responsible for germination inhibition. Gibberellic acid (1.2–1.4 mg/L) further enhanced germination to over 80 % by inhibiting embryonic dormancy and stimulating hormonal pathways. Low-temperature treatment (5 °C) yielded an 83 % germination rate. Mechanical scarification was the most effective method, achieving approximately 90 % germination. In contrast, salinity stress progressively inhibited germination: at 513.3 mM NaCl, germination dropped to 20 % in Khardala and 0 % in Beldia. Seedling growth, including shoot and root length, leaf number and fresh and dry biomass, was also significantly reduced, with root development particularly affected. At 85.5 mM NaCl, root growth declined by 62 % in Beldia and 88.7 % in Khardala, indicating greater salinity sensitivity in Khardala. In conclusion, optimising germination conditions and selecting salt-tolerant varieties are essential for improving the *in vitro* culture of *C. sativa* and producing plants better adapted to saline stress.

Keywords: *Cannabis sativa*; gibberellic acid; hydrogen peroxide; *in vitro* germination; salt stress

Introduction

Cannabis sativa L., also called hemp or marijuana, is the only species in the Cannabaceae family. It is an annual herbaceous plant that is apetalous and mainly dioecious, although it occasionally exhibits a monoecious (hermaphrodite) nature (1, 2). The cannabis plant produces and stores more than 500 active compounds such as terpenes, phenolic compounds, alkaloids, flavonoids and the specific cannabinoids, being the most diverse and extensively studied. Cannabidiol (CBD) and tetrahydrocannabinol (THC) were first detected in 1940 and 1964, respectively (1–5). The THC metabolite is responsible for cannabis' intoxication and delirium effects. Cannabis is well known for its notable therapeutic properties, which include anticancer, anti-inflammatory, anti-bacterial and antidepressant effects, as well as its ability to treat epilepsy and delirium tremens, neuropathic pain and alleviate mood and anxiety disorders (6, 7). In addition, the cannabis plant possesses several non-therapeutic uses, such as the construction, paper and textiles industries. Seeds are a product of the cannabis plant with very important economic and medicinal interests. They are used for vegetal oil extraction, which is exploited in various fields such as food, cosmetic and pharmaceutical industries (7–9).

For years, cannabis cultivation focused on producing fibres or increasing THC content. Although germination is not directly linked to THC production, healthy plants resulting from optimised germination are more likely to produce high THC levels during later growth stages (10, 11). Today, however, the emphasis is on high phytochemical production, highlighting the need for standardised plant material to meet the growing global market demand (6). But several problems have been raised to have a high-quality cannabis plant and therefore derived products with very high added value. Primarily, constraints linked to the plant itself; the cannabis plant is dioecious and characterised by open pollination and high

heterozygosity. Additionally, seed germination is influenced by various abiotic factors that also affect plant development and distribution. Environmental stresses, including fluctuations in precipitation, prolonged dry periods and inappropriate agricultural practices. These factors, along with the coexistence of salinity and drought, create osmotic stress that reduces water availability for seed germination (12). Additionally, the seeds exhibit significant genetic variability (7). These constraints complicate breeding selection, leading to a lack of growth control and traceability, which ultimately affects the quality and quantity of desired secondary metabolites in cannabis (13, 14). Secondly, constraints and requirements linked to the global cannabis market: cannabis has recently experienced a renewed global interest because of its versatile uses and the scientific validation of its medicinal properties. Therefore, there is an exponential increase in demand for cannabis products worldwide for non-psychoactive cannabinoids that can be obtained from industrial production (15). Several previous studies have investigated the factors influencing *C. sativa* germination across diverse environments. Research indicates the effects of varying temperatures on the germination and biochemical profiles of cannabis landraces, revealing significant genetic variability and differential adaptability among varieties (16). Moreover, research developed a standardised protocol for rapid and efficient germination, incorporating the application of gibberellic acid and hydrogen peroxide to optimise *in vitro* performance (17). Finally, research has analysed the germination of cannabis landraces from different geographic regions, highlighting the combined influence of environmental and genetic factors on the germination process (18).

Although the germination and early development of *Cannabis sativa* have been studied in various contexts, few investigations have focused on Moroccan varieties. These varieties exhibit distinctive biological and agronomic traits, such as seed dormancy, germination rate, salinity tolerance and vegetative growth patterns. Studying these varieties, therefore, fills a scientific gap and provides specific data to optimise their *in vitro* and *ex vitro* cultivation. However, research on this versatile plant is limited and biotechnological methods are still in their infancy due to a long-term ban (19–21). The application of biotechnological tools constitutes a promising alternative for the production, selection and conservation of local cannabis species.

In Morocco, throughout history, *C. sativa* was classified as an illegal plant. However, the government has recently legalised and decriminalised its medical use through law no. 13–21 regarding the legal use of cannabis (3). This change has led to a growing interest among researchers and the pharmaceutical industry. This decision opens up significant research opportunities in the field of cannabis and its derivatives in Morocco. In this context, our work focuses on optimising an *in vitro* seed germination protocol for two cultivated cannabis varieties in Morocco: Khardala (introduced variety) and Beldia (landrace). This research will serve as the initial step toward utilising plant tissue culture for applications in micropropagation and, particularly, the extraction of cannabinoid metabolites from cannabis.

To optimise the *in vitro* germination of two Moroccan *Cannabis sativa* varieties, Khardala (introduced) and Beldia (local landrace), five pre-germination treatments (gibberellic acid, hydrogen peroxide, low temperature, darkness and mechanical scarification) were assessed, along with salt stress induced by increasing NaCl concentrations. This is, to the best of our knowledge, the first comprehensive attempt to enhance germination performance in these specific genotypes. The study aimed to define the most favourable conditions for seed germination while evaluating the differential responses of both varieties to abiotic constraints, to identify the one most suitable for *in vitro* propagation under Moroccan conditions.

Materials and Methods

Plant material

The material used as explants to initiate *in vitro* culture consists of seeds from two cannabis varieties: Khardala and Beldia. The seeds were provided by the National Agency of Aromatic and Medicinal Plants in Taounate, Morocco.

Sterilization technique

For sterilisation, the seeds were incubated in 70 % (v/v) alcohol for 1 min before being immersed in a 4.6 % sodium hypochlorite solution for 15 min. Then, they were rinsed three times with sterile distilled water and finally dried on sterile filter paper. All metallic culture tools (tweezers, scalpels, spatulas) were sterilised in a dry oven at 200 °C for 2 hr before each use. During handling under the laminar flow hood, tweezers were further disinfected with ethanol and flame-sterilised before each manipulation.

In vitro culture establishment

After sterilisation, the seeds were placed (after pre-treating or not) in a sterile glass jar containing 25 mL of ½ MS medium solidified with 8 g/L agar-agar and the pH was adjusted to 5.7 before autoclaving at 121 °C and 100 kPa for 20 min (22). The germination temperature was maintained at 25 ± 2 °C. All cultures were incubated under a 16 light/8 photoperiod at a light intensity of 60 µmol m^{-2} sec^{-1} provided by white fluorescent lamps (Sylvania Sylfast SSE T5, Germany) excepting the experiment where we studied the photoperiod effect. A seed is considered to have germinated when the length of the radicle reaches 1 mm or more. All treatments or pretreatments were applied to the seeds of two *C. sativa* varieties. The germination test lasted for one month and the number of seeds studied was specified for each treatment type. The germination percentage evaluation was made after 2 weeks of *in vitro* cultivation. The percentage germination was calculated according to the following Eqn. 1 (15):

$$GP\ \% = \frac{GF}{n} \times 100 \qquad \text{(Eqn. 1)}$$

Where, GP % = germination percentage, GF = the total number of germinated seeds at the end of the experiment and n = the total number of seeds used in the test

Effect of gibberellic acid (GA_3)

To evaluate the effect of gibberellic acid (GA_3) on germination, cannabis seeds were first sterilised and then subjected to a pretreatment consisting of soaking for 48 hr in GA_3 solutions at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 mg/L (23). The prepared solutions, along with Petri dishes containing filter paper, were sterilised and autoclaved. Then, 10 mL of each solution was poured into the Petri dishes. Control seeds were cultured under the same conditions using sterile distilled water instead of GA_3. The test was conducted at ambient temperature, with six replicates per concentration and per variety.

Hydrogen peroxide (H_2O_2) effect

Seeds of both varieties, after sterilisation, were placed in sterile Petri dishes containing sterile filter paper moistened with different concentrations of H_2O_2 (0.5 %, 1 %, 1.5 % and 2 %) (17). Each soaking session lasted 48 hr and was repeated over a total period of one week, with the H_2O_2 solution renewed every 48 hr. No germination was observed during the pre-treatment phase. Control seeds were soaked in distilled water without H_2O_2 under the same conditions. Ten, all cultures were transferred to ½ MS and incubated in a growth room with the conditions mentioned above. Six repetitions per concentration per variety were used. The test was conducted at ambient temperature.

Mechanical scarification effect

The embryos were extracted delicately (to avoid damaging their structure) using a scalpel and fine forceps. A control consisted of intact seeds. All explants (embryos and intact seeds) were cultured in ½MS basal salt medium under culture conditions mentioned above. 16 repetitions/treatment/ variety were used. The test was conducted at ambient temperature.

Temperature effect

To assess the effect of temperature pre-treatment, cannabis seeds were subjected to different temperatures for 24 hr before the sterilisation protocol, including dry cold at 5 °C; dry hot at 35 °C and 40 °C. Then, all cultures were transferred to ½ MS and incubated in the growth room. A control batch at 25 °C was also used. 10 replicates were used for each pre-treatment/variety.

Darkness effect

In this experiment, the first batch of seeds (60) is incubated in ½MS medium under the culture conditions described above (with a photoperiod of 16 hr light and 8 hr dark), while the second batch of seeds (60) is kept in total darkness under the same conditions. The test was conducted at a temperature of 25 ± 2 °C.

NaCl effect

Nutrient media and culture conditions

The culture medium used in this experiment consisted of half-strength MS basal salts (½ MS), supplemented with 1.5 % sucrose, half-strength MS vitamins and solidified with 8 g/L agar-agar (17). Various concentrations of sodium chloride (NaCl) were added: 0, 42.8, 85.5, 171.1, 256.6 and 513.3 mM (24). The pH was adjusted to 5.7 using 1 M NaOH or 1 M HCl before autoclaving at 121 °C for 20 min. Seeds were completely dehusked, while embryos were carefully excised using a scalpel and fine tweezers to avoid injury and eliminate potential tegumentary inhibition. The isolated embryos were then sterilised and subsequently cultured on the prepared media containing the different NaCl concentrations. Each concentration for each variety was replicated ten times. Cultures were maintained under controlled environmental conditions at 25 ±2 °C with 64 % relative humidity and a photoperiod of 16 hr of light and 8 hr of darkness, using white fluorescent lamps providing a light intensity of 60 µmol m^{-2} s^{-1}. The evaluation of salinity effects on *in vitro* culture was carried out after 30 days, based on observations and measurements of germination and seedling growth parameters.

Evaluation of salt stress tolerance *in vitro*

The response to the salt stress is evaluated through the following parameters: germination rate according to Eqn. 1, length of the aerial and root parts, as well as the average number of leaves formed per developed embryo. Relative water content (RWC) of plants was measured as per the standard protocol (25, 26). After determining the fresh weight (FW) determined by the type of organ (roots, aerial parts). Thereafter, the samples were placed in a pre-heated oven at 70 °C for 24 hr to obtain dry weight (DW). The water content is calculated after 1 month of cultivation (Eqn. 2):

$$WC = \left(\frac{Wf - Wd}{Wf}\right) \times 100 \qquad \text{(Eqn. 2)}$$

Data analysis

All results are presented as arithmetic means of multiple replicates, accompanied by standard error (bar variation). The data were analysed using two-way analysis of variance (ANOVA) and means were compared using the Duncan test at a 5 % significance level in SPSS Statistics 22.

Results and Discussion

Effect of gibberellic acid (GA_3)

The impact of gibberellin on seed germination of two industrial hemp cultivars was studied by treating the seeds with different concentrations of GA_3 (0, 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 mg/L). A two-way ANOVA ($P < 0.05$) showed that the hemp variety did not significantly affect germination rate ($P = 0.138$); however, GA_3 pre-treatment and the interaction between them significantly affected the latter. The highest values were observed at 1.2 mg/L (88 ± 5.66 %) and 1.4 mg/L (85 ± 7.33 %) (Fig. 1). It seems that Beldia and Khardala seeds do not show true dormancy (control) and a concentration of GA_3 less than or equal to 0.8 mg/L could improve their germination percentage.

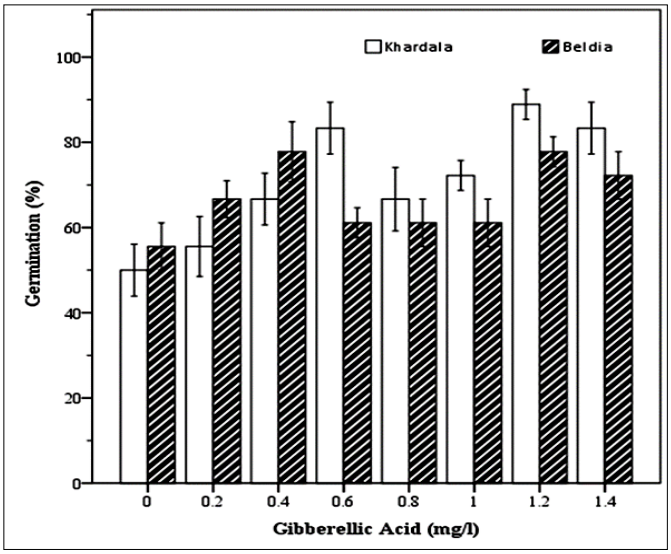

Fig. 1. Effect of gibberellic acid concentration on seed germination of Khardala and Beldia varieties. The variation bar present standard error.

Generally, GA_3 can affect the seed germination. Previous studies have shown that exogenous gibberellic acid promoted seed germination under various abiotic stress conditions in different plants. Gibberellic acid has proven to be highly significant in breaking seed dormancy for these two genotypes, as supported by other researchers (16). It has been reported that the optimum concentration of GA_3 to improve seed germination and to overcome dormancy was 250 and 500 ppm (26, 27). Similarly, two industrial hemp cultivars, 'Yunma 1' and 'Bamahuomas' showed the highest

germination rate when seeds were pre-treated by GA_3 at 400 mg/L and 600 mg/L, respectively (28). These results also indicate that successful germination depends not only on culture conditions but also on other factors such as sowing date, seed maturity, the balance between endogenous and exogenous hormones and especially the genetic variability of the cultivars. Paying particular attention to these aspects can significantly improve germination performance (29). This process enhances seed sensitivity to various environmental factors such as light, nitrates and gibberellins. Moreover, chilling activates genes involved in gibberellin biosynthesis (GA_3ox1) as well as the enzyme that converts inactive gibberellins (GA_9) into active forms (GA_4). It also regulates genes associated with gibberellin concentration and sensitivity, in interaction with abscisic acid. Surprisingly, in the present study, GA_3 improved seed germination of both varieties, Beldia and Khardala, despite its low concentration (1.2 and 1.4 mg/L). Thus, this result indicates that the hemp seeds of both varieties do not present a true dormancy or integumentary inhibition. Indeed, exogenous GA_3 enhanced seed germination, because it seemed to antagonise the effect of abscisic acid (ABA) and increase cell wall plasticity and better water absorption (30). In addition, exogenous GA_3 seemed to induce the activation or synthesis of hydrolytic enzymes such as protease and α-amylase in seeds, thus improving the germination ability of seeds (31–33).

Hydrogen peroxide (H_2O_2) effect

In this experiment, an effective procedure will be developed to optimise the germination percentage of Khardala and Beldia seeds using various liquid pretreatments (0, 0.5, 1, 1.5 and 2 %) of hydrogen peroxide. In Fig. 2, data are presented as mean ± standard error. The application of H_2O_2 concentrations from 0 to 1.5 % was found to positively impact the germination percentage of both varieties. A concentration of 1.5 % yielded a highly significant germination rate in the Khardala variety and also the highest rate in Beldia. However, a concentration of 2 % caused a decrease in germination rate from 77.7 ± 5.66 to 66.66 ± 3.21 % and from 61.11 ± 5.33 to 44.44 ± 6.22 % in Khardala and Beldia, respectively. The two-way ANOVA ($P < 0.05$) analysis showed that H_2O_2 concentration has a highly significant effect on the germination rate ($p = 0.000$) and the variety factor also significantly ($p = 0.03$). However, the interaction between H_2O_2 concentration and variety does not have a significant effect on the germination rate of cannabis seeds ($p = 0.07$). The use of H_2O_2 in *C. sativa* seed germination was previously reported and 1 % was determined as the optimal concentration (34, 35). It is reported that the use of H_2O_2 can facilitate oxidative modifications of proteins to increase the germination potential. Research has proposed two distinct mechanisms for H_2O_2-induced seed germination: the application of exogenous H_2O_2 leads to a reduction in ABA content within the seeds, which is dependent on MAPK (mitogen-activated protein kinase) signalling (35). H_2O_2 negatively affects the transport of ABA from the cotyledon to the embryonic axis, resulting in a significant decrease in ABA content in the seeds (36, 37).

Mechanical scarification effect

Mechanical scarification showed highly significant results ($p < 0.000$), with a germination percentage reaching 98 ± 2.33 and 87.5 ± 3.22 % for Khardala and Beldia, respectively. In contrast, intact seeds displayed considerably lower germination percentage, reaching a maximum of 71.43 % for Khardala and 56.25 % for Beldia (Fig. 3). According to two-way ANOVA ($p < 0.05$) analysis, the scarification and variety effect are significant ($p = 0.000$, $p = 0.021$), however, the interaction between them isn't. These results are in agreement with a previous study, which found that scarification of cannabis seeds led to germination rates up to 90 % within one week (38). Mechanical scarification is an effective preliminary treatment that promotes the germination of various seeds with hard seed coats, such as cannabis seeds (39). Low germination rates observed in some plant species can be attributed to seed coat-imposed inhibition (40). This scarification process helps overcome this issue without damaging the embryo. The scarification process makes seeds more permeable to moisture and facilitates water and oxygen absorption by the embryo. Additionally, it increases the surface area for nutrient absorption and reduces seed coat dormancy, triggering the germination process (40).

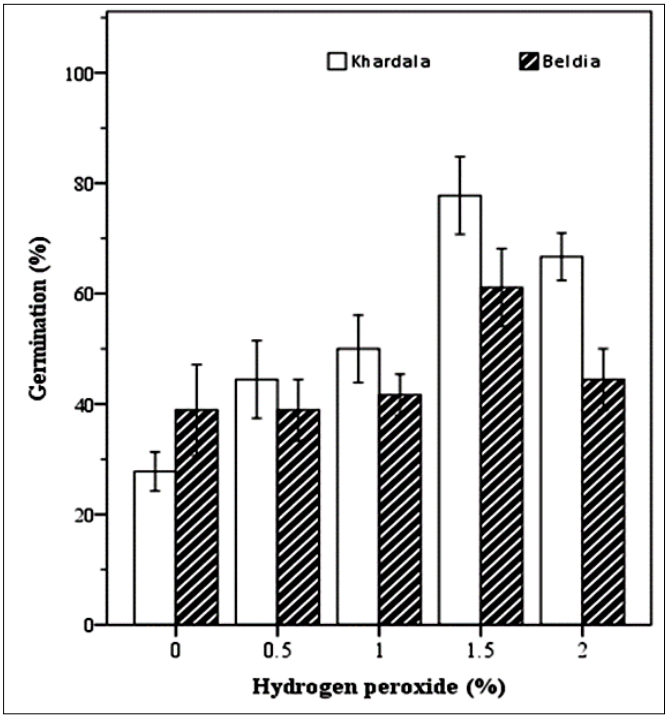

Fig. 2. Effect of hydrogen peroxide on seed germination of Khardala and Beldia varieties. The variation bar present standard error.

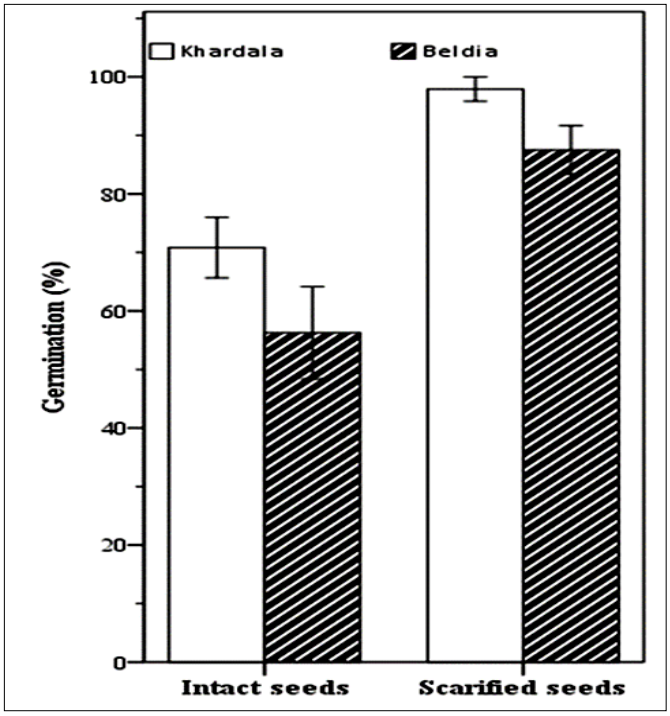

Fig. 3. Effect of mechanical scarification on seed germination of Khardala and Beldia varieties. The variation bar present standard error.

Temperature effect

The temperature variations were found to affect the germination percentage of hemp seeds. At 5 °C, germination percentage was highest, reaching 83.33 ± 8.22 % for both varieties. However, at 35 °C, these values dropped to reach 33.33 ± 2.55 and 41.67 ± 9.23 % for Khardala and Beldia, respectively. At the highest temperature (40 °C), germination percentage was significantly reduced to 8.3 ± 5.11 and 25 ± 4.33 % for Beldia and Khardala, respectively (Fig. 4). The interaction between the two factors, variety and temperature, was found not statistically significant according to ANOVA ($p < 0.05$) analysis ($p=0.000$). It is reported that temperature is a key factor in seed germination, as it affects seed imbibition and the mobilisation of nutrient reserves, which vary with different temperature regimes (41–43). Research has demonstrated that cold stratification at 4 °C enabled 70 % of *Medicago arborea* seeds to break dormancy, significantly improving their germination rate. In contrast, exposure to a higher temperature of 35 °C resulted in germination inhibition (44). This process enhances seed sensitivity to various environmental factors such as light, nitrates and gibberellins. Moreover, chilling activates genes involved in gibberellin biosynthesis (GA_3ox1) as well as the enzyme that converts inactive gibberellins (GA_9) into active forms (GA_4). It also regulates genes associated with gibberellin concentration and sensitivity, in interaction with ABA (45). Elevated temperatures can diminish seed germination potential due to protein damage, disruption of protein synthesis, enzyme inactivation and membrane damage, all of which contribute to poor germination and weak stand establishment and also significantly impair cell division and promote oxidative damage (46). In another study, it is cited that the optimum temperature of cannabis seed germination is 25 °C (47).

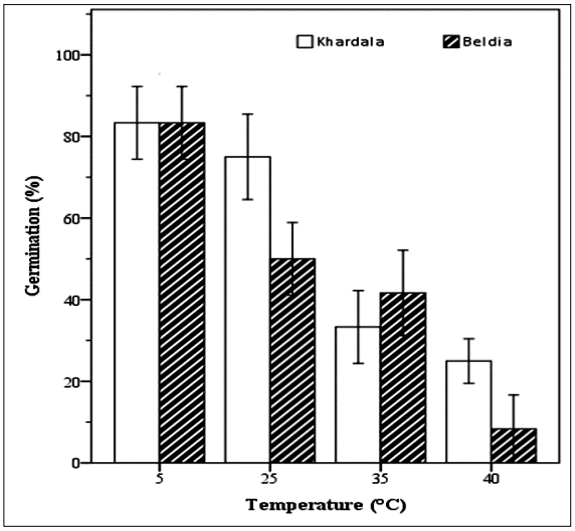

Fig. 4. The temperature effect on seed germination of Khardala and Beldia. The variation bar present standard error.

Darkness effect

The results in Fig. 5 indicate that the germination percentage of the two varieties behaves similarly with respect to photoperiod and total darkness. The germination percentage reached 47.92 ± 6.31 for Beldia and 50 ± 7.22 % for Khardala under photoperiod conditions, but did not exceed 35.41 ± 6.55 and 31.25 ± 6.31 % for Khardala and Beldia, respectively, in complete darkness. According to two-way ANOVA ($p < 0.05$) analysis, the interaction between lighting and variety factors was not statistically significant ($p = 0.875$). Cannabis is reported to be a photophilic plant and the light (intensity and spectrum) influences its growth and development. The previous research showed that light influences the seed germination by ageing on its pigment content (48). This photosensitivity is mediated by a phytochrome, a chromoprotein composed of a chromophore and an apoprotein. This pigment exhibits absorption peaks at 667 nm for the inactive form (Pr) and at 730 nm for the active form (Pfr). In the presence of red light, Pr is converted into Pfr, initiating a cascade of biochemical reactions that lead to radical emergence (49). The effect of light on cannabis seed germination is not well studied, although plants generally rely on light cues for key life cycle stages, including germination. Light can serve as an indicator of favourable conditions for germination, explaining why some seeds specifically require light or darkness, while others can germinate under either condition.

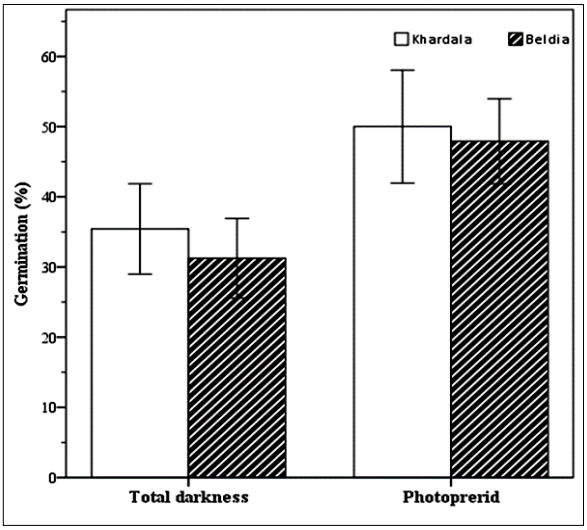

Fig. 5. Effect of total darkness and photoperiod on seed germination of Khardala and Beldia varieties. The variation bar present standard error.

NaCl effect

In vitro embryo germination

Salinity had a negative effect on the *in vitro* germination of isolated embryos in *C. sativa* for both varieties. All embryos grown on media with low concentrations of NaCl (42.8 mM and 85.5 mM) germinated during the first week for both varieties, with a germination rate reaching 100 ± 0.00 % (Fig. 6). However, the saline constraint affects the germination of embryos at a concentration of 171.1 mM NaCl observed in the Beldia variety with a decrease of 60 ± 6.08 % compared to Khardala (Fig. 6 , 7).

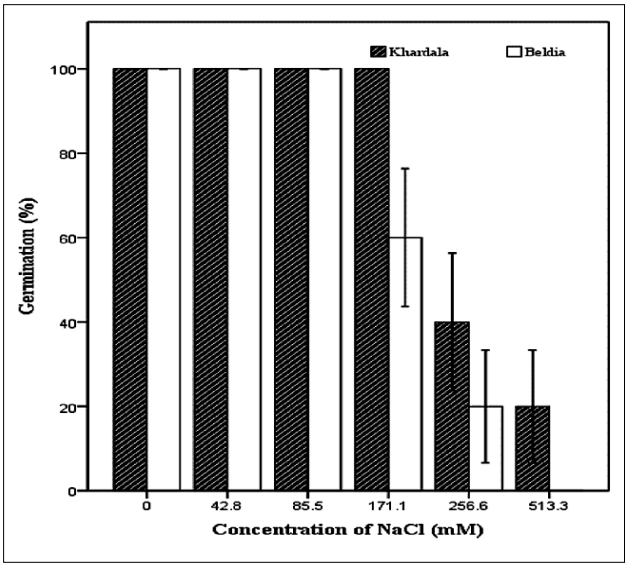

Fig. 6. Germination rate of cannabis seeds of the two varieties Beldia and Khardala in different concentration of NaCl. The variation bar present standard error.

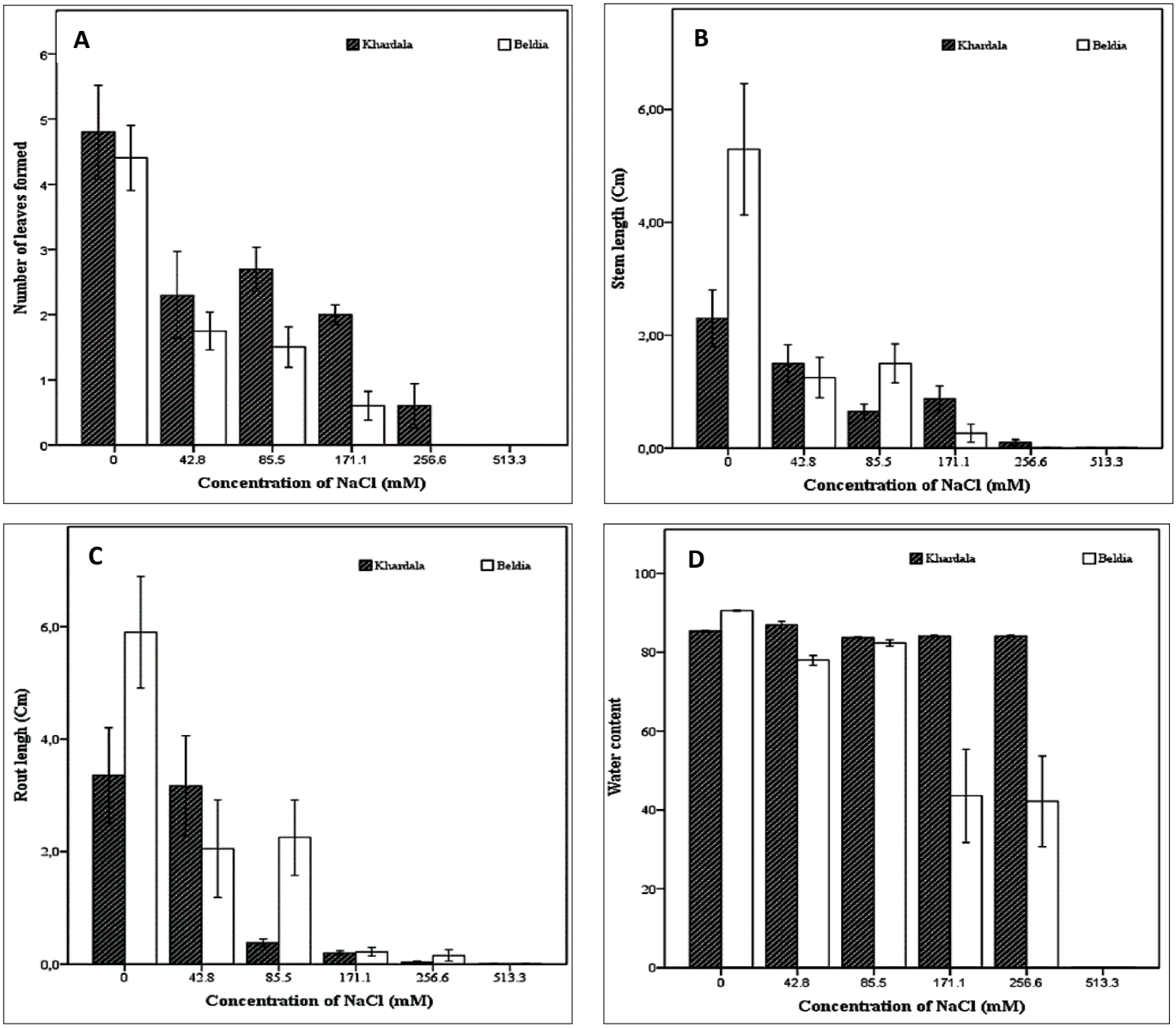

Fig. 7. Variation of different parameters under the effect of NaCl in the two varieties of *Cannabis*. (A) Average number of leaves formed per developed embryo; (B) average length of aerial parts; (C) average length of root parts; (D) water content. The variation bar present standard error.

For a concentration of 256.6 mM NaCl, there is a progressive decrease in germination rate for both varieties, reaching a germination rate of 40 ± 6.08 % for Khardala and 20 ± 6.08 % for Beldia. It can be observed that there is a complete lack of germination for Beldia in the highest saline concentration (513.3 mM), while Khardala still has a germination rate of 20 ± 8.01 %. All the results are highly significant (p = 0.000) for the different NaCl concentrations, but not significant when comparing the two varieties (p = 0.119) using two-way ANOVA (p < 0.05). We can conclude that the germination of the Beldia variety is slightly more sensitive to salt stress than the Khardala variety and that NaCl has led to some inhibition for the Beldia variety. Our results are identical to the results of cannabis seed germination in *ex vitro* for the same concentrations of NaCl found from previous research (50). Generally, it seems that the germination of cannabis seeds in both *ex vitro* and *in vitro* is sensitive to a high level of salts (24).

The growth of *in vitro* seedlings

After one month, the addition of NaCl at all concentrations led to a significant reduction in leaf production per germinated embryo and stem length for both varieties, but this reduction was more significant for the Beldia variety than for the Khardala variety. Thus, NaCl caused a significant shortening of root length, more pronounced for the Khardala variety than for the Beldia variety (Fig. 8). With increasing salt concentration, the effect on growth and development becomes more pronounced. Regarding the leaves, the number increased from 4.40 ± 1.57 to 5 ± 2.25 leaves for both varieties, to 1.50 ± 0.97 leaves for Beldia at a concentration of 85.5 mM, until a complete absence at 256.6 mM. This differed for Khardala, which still had 2.75 ± 1.06 leaves for the same previous concentration, decreasing to 0.66 ± 1.07 leaves for 256.6 mM of NaCl. However, for Beldia, the average stem length was reduced from 5.30 ± 3.67 cm to 1.50 ± 1.08 cm at a concentration of 85.5 mM, further decreasing to 0 ± 0.00 cm at 256.6 mM. This is different for Khardala, where the average stem length was reduced from 2.30 ± 1.58 cm to 0.65 ± 0.41 cm at a concentration of 85.5 mM, further decreasing to 0.10 ± cm at 256.6 mM. For Beldia, the root length decreased from 5.90 ± 3.13 cm to 2.05 ± 2.74 cm at a concentration of 85.5 mM and to 0.15 ± 0.31 cm at a concentration of 256.6 mM. This differs for Khardala, where the root length decreased from 3.36 ± 2.65 cm to 0.65 ± 0.22 cm for the same salt concentration, eventually resulting in the absence of a root system at the highest concentration (Fig. 7A–C). For the RWC, there is no significant difference between the two varieties, but it is clearly visible that the RWC decreases with

Fig. 8. Observation of germinated sheaths in the presence of NaCl. (A) Khardala; (B) Beldia.

increasing salt (Table 1). The presence of salt typically results in a negative impact on the shoot length of seedlings (51, 52). The NaCl led to an inhibition of the cannabis root system (Fig. 7C). The limited development and growth of the vegetative apparatus is reflected in a decrease in the number of leaves observed in both varieties. This reduction in growth is the result at the cellular level of a decrease in the number of cell divisions during abiotic stress (salt and water stress). The reduction in aerial growth observed in *in vitro* seedlings can be explained by disturbances in the levels of certain growth regulators, particularly ABA and cytokinin, induced by salt (53). Indeed, the average number of leaves/explants varies between 4.40 and 4.80 for the control and 0 leaves for the highest concentration due to the accumulation of K^+ and Ca^{2+} in these organs (54). This will lead to competition between Na^+ and Ca^{2+} for the same apoplasmic binding sites. The ratio (root dry matter/aerial dry matter) weakens with increasing saline concentration for both varieties of cannabis. This means that the roots are the most affected. As for the water content, a more significant decrease is observed in Beldia than in Khardala, which means that the hydration state is affected by the salt eaves directly from the embryo (Fig. 7). The Beldia variety, after germination, shows increased resistance to water stress by reacting more quickly. It minimises its growth by reducing the elongation of the aerial surface, decreasing leaf area to limit water loss through evapotranspiration and preserving the roots to the maximum extent to mitigate the effects of salt and prolong its resistance as much as possible. Therefore, the conclusion to be drawn is that the Beldia variety tolerates stress better than the Khardala variety. However, overall, *C. sativa* does not exhibit significant tolerance to salt stress, indicating a low capacity to cope with it. It is known that the harmful effects of salinity are due to osmotic stress, interruption of metabolic activities by excess and ionic imbalance and interference of saline ions with the absorption of essential macro and micronutrients (55). In addition to ionic and osmotic components, salinity stress also leads to oxidative stress by an increase in the amount of reactive oxygen species (ROS), such as reactive oxygen superoxide, hydrogen peroxide and hydroxyl radicals (34).

Conclusion

This study demonstrates that seed germination and early seedling growth in *C. sativa* are significantly influenced by pre-treatments, temperature, photoperiod and salinity. Mechanical scarification yielded the highest germination rates, reaching 98 ± 2.33 % for Khardala and 87.5 ± 3.22 % for Beldia, while GA_3 pre-treatment at 1.2 -1.4 mg/L improved germination to 85-88 ± 5.66 %. Low-temperature pre-treatment (5 °C) also enhanced germination, reaching 83 ± 8.22 % for both varieties. High salinity (up to 513.3 mM NaCl) reduced germination and seedling growth. These quantitative results provide practical guidance for industrial hemp propagation: applying optimal GA_3 concentrations, mechanical scarification, or low-temperature pre-treatment, maintaining moderate culture temperatures (around 25 °C) and controlling salinity in the medium can maximise germination rates and seedling vigour. Such measures can help optimise propagation protocols and improve success in commercial cultivation programs.

Table 1. Effect of NaCl concentration on fresh weight, dry weight and root-to-shoot dry weight ratio in two *Cannabis sativa* varieties

Varieties	Concentration of NaCl (mM)	Weight fresh (mg)	Weight dry (mg)	Weight dry (r)/ Weight dry (a)
Beldia	0	141.00 ± 19.82	13.40 ± 1.77	0.22 ± 0.03
	42.8	34.50 ± 19.82	7.75 ± 1.77	0.11 ± 0.03
	85.5	47.00 ± 19.82	8.50 ± 1.77	0.06 ± 0.03
	171.1	32.80 ± 19.82	9.00 ± 1.77	0.07 ± 0.03
	256.6	27.2 ± 19.82	8.25 ± 1.77	0.03 ± 0.03
	513.3	0.00 ± 19.82	0.00 ± 1.77	0.00 ± 0.03
Khardala	0	94.20 ± 18.48	13.80 ± 2.55	0.19 ± 0.02
	42.8	114.67 ± 18.48	16.00 ± 2.55	0.07 ± 0.02
	85.5	93.50 ± 18.48	15.25 ± 2.55	0.03 ± 0.02
	171.1	72.25 ± 18.48	11.50 ± 2.55	0.05 ± 0.02
	256.6	22.00 ± 18.48	6.00 ± 2.55	0.00 ± 0.02
	513.3	0.00 ± 18.48	0.00 ± 2.55	0.00 ± 0.02

Values are expressed as mean ± standard error (SE).

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Authors' contributions

MT carried out the conceptualisation, methodology, investigation and data curation and drafted the original manuscript. AN contributed to the conceptualisation, methodology and writing-original draft and performed the manuscript review, editing and project supervision. HEA participated in the methodological design. SM and IT performed the software validation and formal analysis. KS, IB and IT contributed to the review and editing of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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