The effects of proline on in vitro proliferation and propagation of doubled haploid sugar beet (*Beta vulgaris*)

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Abstract: Doubled haploid induction is one of the available methods normally used for sugar beet breeding. Gynogenic haploid explants of sugar beet induced with 1 or 2 mg L^{-1} 6-benzylaminopurine were treated with 5 g L^{-1} colchicine, then subcultured on a solidified MS medium plus 0.2 mg L^{-1} kinetin. Colchicine doubled the chromosome number of 27.7% of the treated haploid explants. With the aim of increasing the number of doubled haploid explants, the effects of five levels of proline (0.0, 0.1, 0.2, 0.3, or 0.4 mM) on the explants' proliferation, propagation, and shoot length were compared. With a large effect size (ES), proline at 0.3 mM induced the highest amount of proliferation, while proline-free medium resulted in the lowest amount of it. The highest propagation rates were observed for the explants treated on media with 0.2 and 0.3 mM proline (very large ES). Proline at 0.3 mM induced the shortest shoots (medium ES). A very strong positive correlation between proliferation and propagation, a moderate negative correlation between proliferation and length, and a strong negative correlation between propagation and length were observed. For the first time our results show beneficial effects of proline on in vitro proliferation and propagation of sugar beet.

Key words: Proline, propagation, proliferation, doubled haploid, *Beta vulgaris*

1. Introduction

Sugar beet (*Beta vulgaris*) is an economically valuable crop (Řezbová et al., 2016). It is a biennial and allogamous species. Due to the former, its conventional breeding is time-consuming, while the latter makes it recalcitrant to the implementation of a universally applicable in vitro method. Since conventional breeding of sugar beet is not efficient, research and breeding programs favor biotechnological techniques over conventional ones. Generally, biotechnological techniques require in vitro methods to provide starting material for research and breeding. Sugar beet breeding has benefitted from several tissue culture techniques (Mezei et al., 2006). Despite this fact, in vitro techniques for sugar beet still lag behind those for many major crops (Maluszynski et al., 2003). In addition, while for major crops, e.g., corn, barley, and rye, haploid and doubled haploid production through in vivo or in vitro methods is very efficient, for sugar beet it is not. Gynogenesis (haploid embryo induction through unfertilized cells of the female gametophyte) is one of the in vitro techniques that has greatly served sugar beet breeding. However, this technique has not been adequately efficient (Aflaki et al., 2017).

Despite recent achievements and advances in in vitro tissue culture of numerous plants, for sugar beet it is not very productive (Gürel and Gürel, 2013). It is not amenable to routinely applied haploid induction methods (Aflaki et al., 2017). Allogamous species subjected to in vitro techniques suffer from inter- and intragenotypic variations (Gürel, 1997). Due to the variations, different genotypes' responses to the same method are diverse (Pazuki et al., 2018a). Therefore, one cannot always propagate the desired sugar beet genotypes sufficiently and efficiently. Micropropagation has been used for sugar beet cloning (Klimek-Chodacka and Baranski, 2013). For instance, protoplast fusion (Gürel et al., 2002), chromosome doubling, and even transformant regeneration produced after performing cutting-edge research (Karimi-Ashtiyani et al., 2015) may take advantage of follow-up micropropagation.

Cytokinins (CKs) and auxins are plant growth regulators (PGRs) are mostly used for in vitro plant propagation. In sugar beet micropropagation, CKs are critical for inducing proliferation and its follow-up propagation (Gürel et al., 2008). However, the concentration of CKs needs to be fine-tuned for this species; otherwise, applying the hormone

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at higher concentrations can result in callogenesis, hyperhydricity, difficult rhizogenesis, necrosis, abnormal growth, and inefficient acclimation (Pospíšilová et al., 2000; Klimek-Chodacka and Baranski, 2013; Górecka et al., 2017; Pazuki et al., 2018a). Therefore, to maximize efficiency, CKs should be applied at an optimum level to increase proliferation/propagation while minimizing the side effects.

Proline, a multifunctional amino acid, has diverse and marked effects on plants (Szabados and Savoure, 2010). Proline accumulation is a common response of many plant species to environmental stresses, including flooding, drought, salinity, UV irradiation, high and low temperature, heavy metals, and oxidative stress (Franck et al., 2004; Dörffling et al., 2009; Aksakal et al., 2017; Per et al., 2017). Proline accumulation diminished reactive oxygen species (ROS) levels in wheat by increasing peroxidase and catalase levels, and thus protected it from salinity stress (Manjili et al., 2012). The addition of amino sugars and proline (17.36 mM) together to in vitro medium increased the incidence of somatic embryogenesis by 4- to 5-fold in *Cichorium* (Couillerot et al., 2012). In a comparative study on rice, proline supplementation (24.32 mM) to in vitro media increased scutellar callus fresh and dry weights more than any other supplemented amino acids did (Pazuki et al., 2015). Addition of proline (2.15 mM) to begonia pretreatment medium significantly improved the efficiency of frozen shoots surviving for cryopreservation (Burritt, 2008).

Sugar beet is a rosette explant, for which in vitro proliferation is an indispensable prerequisite for propagation. However, sometimes proliferated explants show limited capability to propagate. Normally, a short rosette explant is easier to manipulate and subculture in vitro.

The protective role of proline under biotic and abiotic stress conditions has been demonstrated in many studies (Szabados and Savoure, 2010). The effects of two different CKs have been previously investigated to efficiently improve sugar beet in vitro propagation (Pazuki et al., 2017). To the best of our knowledge, the role of exogenous proline in in vitro proliferation and propagation has not been studied. Therefore, we examined whether proline could improve the proliferation and propagation of doubled haploid sugar beet explants.

2. Materials and methods

2.1. Plant material
Inflorescences (10 ± 2 cm in length) of a diploid (2n = 2x = 18) self-fertile sugar beet (*B. vulgaris*) genotype (SG3) were collected in June (Sugar Institute, Etimesgut, Ankara, Turkey). The inflorescences were either used fresh or pretreated for 1 week at 4 °C in a refrigerator. After

removing the bracts, the spikes were sterilized with a 70% alcohol solution for 5 min; then, without rinsing, they were sterilized further with a sodium hypochlorite solution (6%–14% active chlorine) diluted in distilled water (DW) (for 100 mL of the solution: 23 mL of NaOCl + 77 mL of DW, plus 4 drops of Tween-20). After manually shaking for 30 min, the explants were rinsed with DW three times.

2.2. Gynogenesis medium composition and incubation conditions
Under a stereomicroscope, using forceps and a scalpel, ovules were detached from the ovaries and cultured on 90-mm disposable petri dishes. Gynogenesis medium was composed of MS (Murashige and Skoog, 1962) salts and vitamins, 100 g L^{-1} sucrose, and 2.8 g L^{-1} Phytagel. In the PGR treatments, in addition to the control (hormone-free: HF), 1 or 2 mg L^{-1} BAP was used (see Pazuki et al., 2018a). The pH was adjusted to 5.8 before autoclaving. The dishes containing ovules were kept in a growth chamber with a 16-h photoperiod at a constant temperature of 24 ± 2 °C.

2.3. Diploidization
Chromosome set doubling was done using a modified gynogenesis medium previously explained (Pazuki et al., 2018b), in which 2 g L^{-1} GELRITE was used instead of 2.8 g L^{-1} for solidification. A 2% solution of colchicine was sterilized using a 22-μm filter. After cooling the autoclaved medium, the solution was mixed with it to make 5 g L^{-1} doubling medium. The haploid gynogenic plantlets were consecutively grown on 45 ± 5 mL of media in Magenta boxes containing MS medium supplemented with 30 g L^{-1} sucrose and 0.5 mg L^{-1} BAP, then on 30 g L^{-1} sucrose, hormone-free, and solidified with 2.8 g L^{-1} Phytagel, and finally on 10 g L^{-1} sucrose, 0.05 mg L^{-1} BAP, and 0.5 mg L^{-1} kinetin, solidified with 3 g L^{-1} Phytagel. The proliferated plantlets with 3–7 leaves were subcultured on colchicine-supplemented medium. The plantlets were treated for 5 min. After doubling treatment, the plantlets were removed from the medium and directly subcultured on the proline-free proliferation and propagation medium. They were propagated for 2 months and then they were subcultured on proline-supplemented media.

2.4. Proline treatment
After doubling the chromosome number, all the explants were propagated, randomly segregated, and subcultured on 45 ± 5 mL of medium in Magenta boxes containing MS medium supplemented with 10 g L^{-1} sucrose and 0.2 mg L^{-1} kinetin and solidified with 6.5 g L^{-1} Phytagel. This medium was chosen based on a previously conducted experiment to control the hyperhydricity of sugar beet in vitro explants (Pazuki et al., 2017). The explants, which were propagated, were divided into new explants with three leaves using a scalpel and forceps. Making the explants with three leaves prevented conducting a biased experiment. Then they

were subcultured and propagated monthly on the same medium. After 3 months, all the doubled haploid explants were subcultured on the same media (proline-free media), plus four media supplemented with 0.1, 0.2, 0.3, or 0.4 mM proline. The pH of the media was adjusted to 5.8 before adding the solidifying agent, and then they were autoclaved at 121 °C and 100 kPa above atmospheric pressure for 15 min. After autoclaving, filter sterilized (22-μm) aqueous solutions of proline were mixed with the media.

2.5. Ambient conditions
The explants were incubated in a growth chamber with a 16-h photoperiod at a constant temperature of 24 ± 2 °C with 50 ± 5 μmol m^{-2} s^{-1} radiation from cool white fluorescent tubes (Master TL-D 840, Philips, Pila, Poland), at relative humidity of $70 \pm 10\%$.

2.6. Flow cytometry analysis
Sugar beet and common vetch (*Vicia sativa*) leaf tissues were simultaneously chopped with a razor blade in a plate containing 400 μL of extraction buffer of CyStain UV Precise P (Partec, Münster, Germany). The nuclei suspension was passed through a CellTrics 30-μm filter into a glass tube. Next, 1600 μL of 4',6-diamidino-2-phenylindole (DAPI) was added to each glass tube and staining proceeded for a few minutes at room temperature. The samples were analyzed using a Partec CyFlow Space flow cytometer. To estimate the absolute value of DNA content (1C) for each sample, Doležel and Bartos's (2005) formula was calculated: [(G1 peak mean of *B. vulgaris* / G1 DNA content (2C) of *V. sativa*)] × G1 peak of *V. sativa*.

2.7. Mitosis analysis
Young leaves of haploid and doubled haploid in vitro plantlets were treated with a 2×10^{-3} M aqueous solution of 8-hydroxyquinoline for 3 h at room temperature. Then they were fixed in a freshly prepared 96% ethanol:hydrochloric acid solution (2:1 v/v) for 15 min, after which the leaves were rinsed with distilled water and then kept in it. A small piece of the leaf tissue was transferred to a drop of 3% orcein in 45% acetic acid on a slide. The tissue was gently pressed under a coverslip to squash it. The coverslip was pressed by fingertip from one side to the other to spread the metaphase plates. The chromosomes were counted under a light microscope.

2.8. Observation
After 3 weeks growing on media containing or not containing proline, all leaves grown from each explant were counted to calculate and analyze the effects of treatment on proliferation. In addition, the number of shoots propagated from each treated explant was recorded. Shoot length of the treated explants was also measured.

2.9. Experimental design and statistical analysis
The experiment was carried out in a completely randomized design with 5 treatments and 15 replicates.

The observation records were tested for assumptions of normality and homogeneity of variances using Shapiro–Wilk and Lilliefors-corrected Kolmogorov–Smirnov tests (S-W and K-S tests), and Levene's test, respectively. Gynogenesis records were analyzed using one-way ANOVA and a follow-up analysis of Tukey's HSD test (P < 0.05). The results from the treatment effects on proliferation and shoot length were analyzed using Welch's adjusted *F* ratio for one-way ANOVA; then a Games–Howell (G-H) post hoc analysis test was run (P < 0.01). For propagation (producing new shoots), the result was subjected to a Kruskal–Wallis (K-W) test, and the means were compared using the Bonferroni-corrected Dunn's post hoc test to protect against inflation of the familywise type I error rate resulting from the K-W test (P < 0.01). To estimate unbiased effect size (ES) of the independent variables, omega-squared (ω^2), adjusted omega-squared ($est.\omega^2$), and epsilon-squared (ε^2) values were computed (Cohen, 1988; Field, 2013). In addition, Kendall's tau-b (τ_b) correlation coefficient was computed to estimate the bivariate correlation coefficient between proliferation, propagation, and shoot length (Howell, 2012). SPSS 23.0 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analysis and graph drawing.

3. Results and discussion
It was observed that BAP treatments produced different gynogenic embryos, and proline was effective in inducing explants for high quality proliferation. However, to estimate the actual effects of independent variables, the results were subjected to statistical analyses. Assumptions for all the statistics were investigated to ensure the accuracy of analyses.

3.1. Haploid and doubled haploid production
Different gynogenesis rates were induced using the varied BAP concentrations. The result for haploid embryo induction was tested for assumptions of normality and homogeneity of variances. S-W, Lilliefors-corrected K-S, and Levene's tests were all met ($F(2, 6) = 0.507$, P = 0.626). ANOVA and Tukey's post hoc test were conducted to evaluate significant differences between the means and to compare them (P < 0.05). The analysis result was significant for ANOVA and the follow-up test ($F(2, 6) = 8.376$, P = 0.018, $\omega^2 = 0.95$). BAP at 1 mg L^{-1} induced the highest gynogenic embryos (M = 38.1, SD = 7.28, 95% CI [12.09, 48.27]), while hormone-free medium induced the lowest (M = 19.03, SD = 4.75, 95% CI [7.23, 30.83]) (Figure 1A). Differential gynogenic response rates were also reported by other research groups that investigated the effect of BAP on sugar beet gynogenic embryo induction (for a recent review, see Aflaki et al., 2017). While most of the studies on sugar beet gynogenesis resulted in low response rates (Eujayl et al., 2016; Aflaki et al., 2017),

others produced high levels of gynogenesis response up to 45.5% (Pedersen and Keimer, 1996). To avoid the pitfall of relatively inefficient gynogenesis in sugar beet and to improve the efficiency of the technique, some research programs benefitted considerably from gynogenic embryo induction of highly responsive doubled haploid (Hansen et al., 2000) or male sterile donor plants (Svirshchevskaya and Doleze, 2000). The efficiency of doubling for the present study was 27.7% of treated haploid explants. In comparison with others' attempts at sugar beet doubled haploid production (Eujayl et al., 2016), the efficiency of the present method is higher. The ES of the treatments on gynogenesis rate is large, which is notable for recalcitrant plants. The relatively high response of gynogenesis for the present experiment could be ascribed to the hormonal treatment, the genotype, and seasonal effects (see Pedersen and Keimer (1996) and Aflaki et al. (2017) for an extensive review of the assumed independent variables' effects).

3.2. Cytogenetics

The explants were treated on a solidified medium containing 5 g L^{-1} colchicine for 5 min. By using Doležel and Bartos's (2005) formula, G1 DNA contents of haploid and doubled haploid explants were calculated. For haploids it was [(109.53 / 523.29)] × 3.65 = 0.763 pg; for doubled haploids it was [(214.9 / 511.37)] × 3.65 = 1.533 pg (Figures 1B and 1C). Cytogenetic analysis confirmed haploid and doubled haploid numbers of chromosomes for the plant materials. Nine chromosomes for haploid and 18 for doubled haploid were counted under a light microscope, as well. The records were in agreement with previous cytological studies on *B. vulgaris* (Barow and Meister, 2003; Sliwinska et al., 2005; Weber et al., 2010; Castro et al., 2013). Induced doubled haploid explants were used to examine proline's effects on proliferation.

3.3. The effect of proline on shoot proliferation

Shoot proliferation was tested by S-W, Lilliefors-corrected K-S, and Levene's tests. The assumption of normality was met; however, the assumption of homogeneity of variances was not met ($F(4, 70) = 8.932$, $P < 0.001$). Therefore, Welch's adjusted F ratio analysis and the G-H post hoc test ($P < 0.01$) were used for comparison of the treatment means. The effects of proline treatments on mean rates
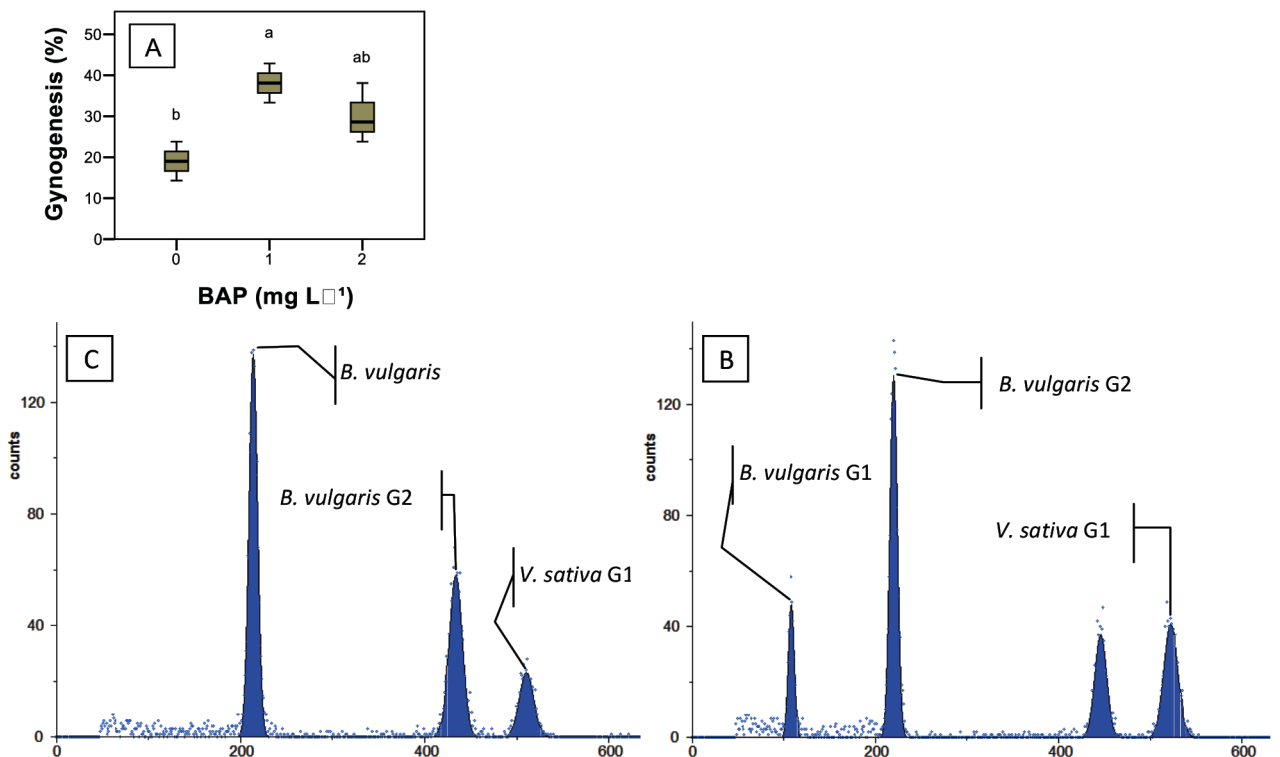

Figure 1. Gynogenesis and ploidy level analysis of sugar beet (*B. vulgaris*). A) Three hormonal treatments, i.e. hormone-free (HF) or 1 or 2 mg L^{-1} BAP, were applied to induce gynogenic embryos from a sugar beet genotype. B) A flow cytometry histogram of haploid and C) doubled haploid sugar beet. The heavy black line inside each box marks the 50th percentile, or median, of that distribution. The lower and upper hinges, or box boundaries, mark the 25th and 75th percentiles of each distribution, respectively. Whiskers mark the largest and smallest observed values that are not statistical outliers.

of proliferation were statistically significant (Welch's F(4, 33.942) = 487.099, P < 0.001, $est.\omega^2$ = 0.963). The G-H test indicated that all the treatments were statistically different from each other, except for 0.1 and 0.4 mM proline (SD = 0.394, P = 0.986, 95% CI [−1.62, 1.22]). While proline at 0.3 mM induced the highest amount of proliferation (M = 42.4, SD = 3.72, 95% CI [40.34, 44.46]), proline-free medium resulted in the lowest amount of it (M = 5.07, SD = 1.22, 95% CI [4.39, 5.74]) (Figures 2A and 2B). Sugar beet tissue culture still suffers from a lack of efficient protocols. Sugar beet doubled haploid production through androgenesis has been attempted many times (Aflaki et al., 2017). Although all the androgenic attempts failed, recently androgenesis from sugar beet was tried by Górecka et al. (2017). In spite of inducing dozens of androgenic embryoids and calli, none of them regenerated or even survived. A genotypic effect on failure was not refuted and the inefficiency of the protocol was not denied (Górecka et al., 2017). The treated explants of haploid and doubled haploid can be decreased by necrosis (Klimek-Chodacka and Baranski, 2013). As a result, the net proliferation and subsequent propagation may be highly decreased. Putnik-Delic et al. (2013) studied proline accumulation in sugar beet plants/explants grown under drought stress in a greenhouse or in vitro. Under drought conditions, drought-tolerant genotypes accumulated higher amounts of proline than intolerants did. In optimum in vitro conditions, tolerant genotypes produced higher numbers of axillary buds than intolerant ones did, although both of them accumulated the same amount of proline. In the present experiment, since the explants treated with proline were not in stressful conditions, assumingly they mostly utilized proline not in a stress reaction process but in growth and proliferation. The ES of proline on the dependent variable was large enough to be taken into consideration for future research programs.

3.4. The effect of proline on shoot propagation

Mean propagation rates of the treated explants were examined for assumptions of normality and homogeneity of variances. S-W and Lilliefors-corrected K-S tests showed that the results violated the corresponding assumption; however, the assumption of homogeneity was met after running Levene's test (F(4, 70) = 2.463, P < 0.053). The treatment effects on propagation were compared using one-way ANOVA on ranks to guard against the bias of repeated testing effects. The mean ranks for 0, 0.1, 0.2, 0.3, or 0.4 mM proline were 19.07, 27.47, 56.37, 63.83, and 23.27, respectively. A K-W chi-square test showed that the main effect of proline on propagation was statistically significant (χ^2 (4, N = 75) = 56.23; P < 0.001). To reduce the chances of obtaining false-positive results, a step-down follow-up analysis using the Bonferroni-corrected Dunn's

post hoc test (P < 0.01) was conducted. The test indicated that media containing 0, 0.1, and 0.4 mM proline induced the least shoot propagation (M = 1.4, SD = 0.632, 95% CI [1.05, 1.75]; M = 1.8, SD = 0.561, 95% CI [1.49, 2.11]; M = 1.6, SD = 0.632, 95% CI [1.25, 1.95]), while 0.2 and 0.3 mM proline induced the most (M = 3.8, SD = 0.915, 95% CI [3.36, 4.37]; M = 4.8, SD = 1.146, 95% CI [4.17, 5.43]) (Figures 2A and 2C). The ES of the independent variables estimated with epsilon-squared was ε^2 = 1.0. Although incorporating CKs into sugar beet in vitro culture medium generally induces propagation, at the same time it can lead to hyperhydricity and necrosis (Klimek-Chodacka and Baranski, 2013; Pazuki et al., 2017). However, our observation indicated that proline-treated explants were all free of those symptoms. Sugar beet is not a very amenable species to in vitro tissue culture (Gürel et al., 2008). Ivic-Haymes and Smigocki's (2005) results suggested that in molecular breeding and improvement programs of sugar beet, a large number of individual plants needed to be screened to identify highly proliferating and propagating ones. They recorded 0.0 to 8.3 ± 1.1 shoot propagation after 7 weeks from 8 sugar beet genotypes, including a model, highly regenerative tissue cultured clone, REL-1. Moreover, in Ivic-Haymes and Smigocki's (2005) experiment, approximately 10% of the regenerants could not be rooted. However, in the present study, the explants treated with 0.2 and 0.3 mM proline produced the highest number of shoots (3.87 ± 0.915 and 4.8 ± 1.146, P = 1.000) after 3 weeks. In addition, all the explants were rooted after 5 ± 2 weeks. Putnik-Delic et al. (2013) observed that drought-tolerant genotypes accumulated higher amounts of proline in drought conditions, and, at the same time, they produced more shoots. Our observation in optimum in vitro conditions indicated that proline between 0.2 and 0.3 mM induced the highest rates of propagation. Proline's ES on propagation was very large and thus applying 0.2 and 0.3 mM proline can be used in future research or breeding programs. However, propagation rates at lower or higher concentrations (0.1 mM or 0.4 mM) were statistically similar to that of proline-free medium (Figure 2C).

Proline increases plants' tolerance to abiotic stresses. Dehydration represses proline catabolism by proline dehydrogenase, whereas rehydration triggers the opposite reaction (Szabados and Savoure, 2010). Hyperhydricity can result from higher than optimum levels of CK. Water accumulates extensively in the apoplast of hyperhydric leaves (van den Dries et al., 2013). As a result, flood-stressed plants generate reactive oxygen species (ROS) (Tian et al., 2017). Proline can scavenge ROS and act as a singlet oxygen quencher (Szabados and Savoure, 2010). Abnormal leaf morphogenesis was observed in *Arabidopsis*

plants expressing an antisense of pyrroline-5-carboxylate synthetase (Nanjo et al., 1999). The CK used in the present experiment left plants prone to hyperhydricity (Pazuki et al., 2017). However, supplementing proline resulted in none of the treated explants showing hyperhydricity symptoms. Proline is usually considered a protective metabolite. In a hypersensitive response via ROS signals, proline triggers programmed cell death and apoptosis. However, under certain conditions, exogenous proline can be deleterious to plants and exposes them to ROS (Szabados and Savoure, 2010). The fewer shoots propagated from the explants treated in 0.4 mM proline may be explained by the stress triggered by ROS signals (Verbruggen and Hermans, 2008).

3.5. The effect of proline on shoot length

Data recorded for the length of shoots at the end of the treatment were evaluated using S-W, Lilliefors-corrected K-S, and Levene's assessments. The normality assumption was met, whereas the assumption of homogeneity of variance was not ($F(4, 70) = 3.407$, P = 0.013). A Welch's adjusted F ratio analysis and G-H post hoc test (P < 0.01)

were used for mean comparisons. The effects of the AA treatment on the shoot lengths were statistically significant (Welch's $F(4, 33.404) = 45.447$, P < 0.001, $est.\omega^2 = 0.703$). Proline at 0.3 mM induced the shortest shoots (M = 1.467 cm, SD = 0.255, 95% CI [1.325, 1.608]). In contrast, proline at 0.1 mM induced the longest shoots (M = 2.833 cm, SD = 0.356, 95% CI [2.636, 3.03]) (Figures 2A and 2D). Tsai and Saunders (1999) examined higher concentrations of proline in a sugar beet model clone, REL-1. The clone was a diploid self-fertile, superior regenerator of shoots from leaf callus. They investigated the effects of 30 and 60 mM proline and several other organic and inorganic nitrogen sources on the fresh weight of proliferated explants. Based on their observations, proline was one of the worst nitrogen sources for weight gain, although all the treatments resulted in lighter fresh weight than MS medium. The lighter weights of the explants reported by Tsai and Saunders (1999) could be due to the toxicity of proline at megadoses (30 and 60 mM) applied exogenously (Verbruggen and Hermans, 2008). In the present experiment, by applying lower concentrations of proline (0.1–0.4 mM), the optimum and
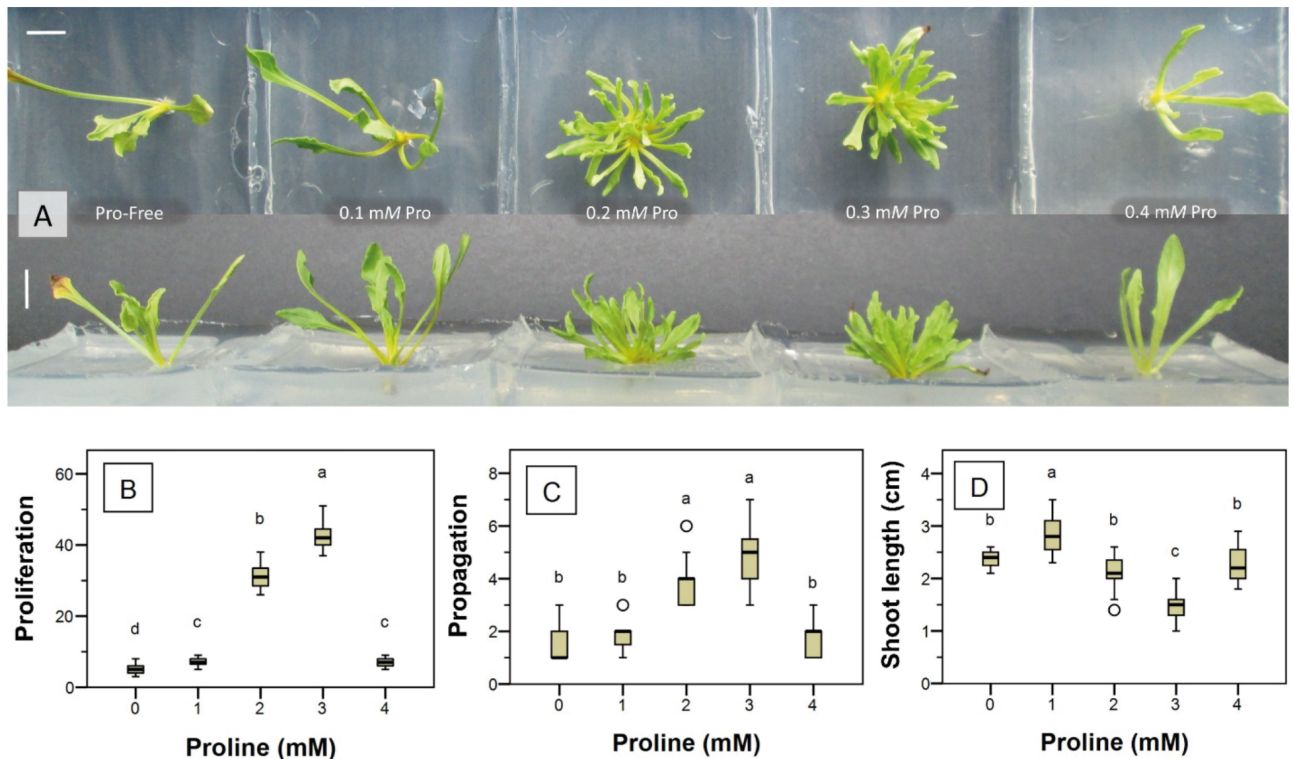

Figure 2. Effects of different proline concentrations on proliferation, propagation, and shoot length. A) Effects of 0, 0.1, 0.2, 0.3, and 0.4 mM proline on the dependent variables are shown (bar = 1 cm). B) Treatment effects on proliferation, C) propagation, D) and shoot elongation. The heavy black line inside each box marks the 50th percentile, or median, of that distribution. The lower and upper hinges, or box boundaries, mark the 25th and 75th percentiles of each distribution, respectively. Whiskers mark the largest and smallest observed values that are not statistical outliers.

the high threshold concentrations of proline for sugar beet in vitro tissue culture and propagation were determined. The short length of shoots grown on 0.3 mM proline might arise from the fact that new leaves act as sinks for nutrients and proline supplemented to the media, thus preventing shoots from growing longer. Proline's ES on shoot length was large.

3.6. The correlations between dependent variables

A Kendall's tau-b correlation was run to determine the relationship between proliferation, propagation, and shoot length, regardless of the independent variables. There was a very strong, positive, and significant correlation between proliferation and propagation (τ_b = 0.822, SE = 0.027, n = 75, P < 0.001) (Figure 3A). Between shoot proliferation and length, there was a moderate, negative, and significant correlation (τ_b = –0.565, SE = 0.061, n = 75, P < 0.001) (Figure 3B). The correlation between shoot propagation and length was strong, negative, and significant (τ_b = –0.601, SE = 0.054, n = 75, P < 0.001) (Figure 3C). Since sugar beet in tissue culture medium generally is a rosette plant, it is normally propagated by dividing proliferated shoots. However, sometimes proliferation is not in a favorable pattern to propagate more propagules (Pazuki et al., 2017). Among some nonstructural carbohydrates and osmoprotectants, in comparison with roots, proline concentration in leaves (as a sink) increased more than any other ones (Hagedorn et al., 2016). Apparently in the present experiment, an exogenous source of proline was utilized for proliferating leaves as a sink rather than increasing shoot length (Perchlik and Tegeder, 2017). However, proline at megadoses resulted in the smallest expansion of the leaf disc, highest percentage of disc callusing, and lower shoot regeneration (Tsai and Saunders, 1999). By computing a correlation between proliferation

and propagation, we showed that the association between the two dependent variables is very strong and positive. Propagation had a more negative association with shoot length than with proliferation, which suggests that an increased number of propagules may result in shorter shoots.

In conclusion, sugar beet is a recalcitrant plant to in vitro tissue cultures and such recalcitrance makes it a relatively inefficient species for biotechnological methods of breeding. Since a tissue-cultured sugar beet explant grows in sterile conditions, it does not face biotic stresses. However, abiotic stresses may affect the explant. We investigated the effects of four proline concentrations on the proliferation, propagation, and shoot length of sugar beet doubled haploid explants. By applying 0.1–0.4 mM proline, we observed that proline at 0.4 mM is deleterious to the in vitro growth of sugar beet. Proline at 0.3 mM induced more proliferation while both 0.2 and 0.3 mM proline induced statistically similar propagation rates. Although proline at 0.1 mM was less favorable, it yielded better proliferation and propagation rates in comparison with proline-free medium. The longest shoots were produced by 0.1 mM proline, while the shortest ones grew on the medium with 0.3 mM proline. To increase proliferation and propagation rates of in vitro cultured explants of sugar beet, proline supplementation to the medium is highly recommended. The results indicated that exogenous application of proline for sugar beet in vitro growth is stimulating below 0.4 mM. In addition, the explants redirected their growth to increase proliferation, but it was at the expense of explant height. For the first time, in the present paper, we provided data to suggest that proline at certain levels can be efficient for in vitro growing of sugar beet explants.
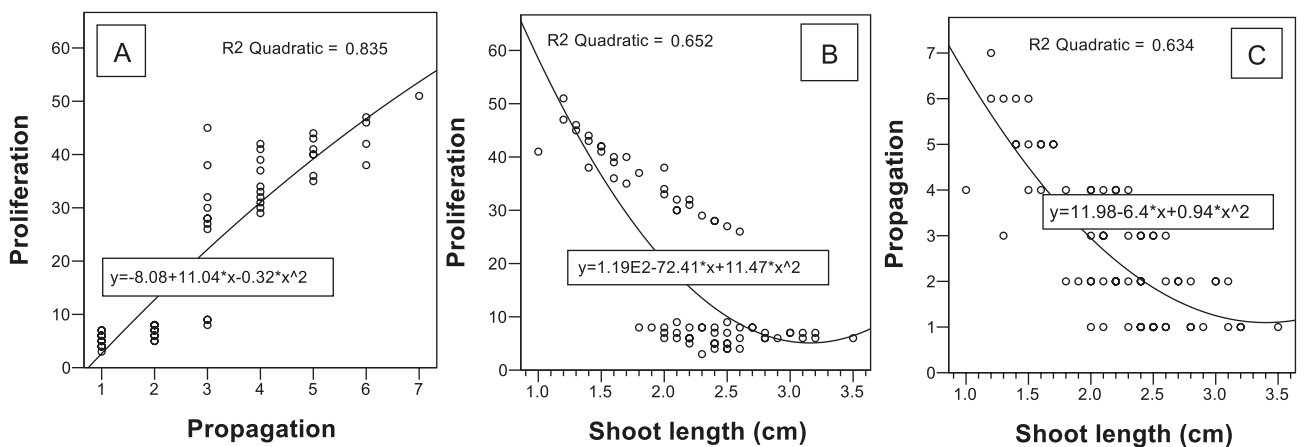

Figure 3. Bivariate correlation coefficient between proliferation, propagation, and shoot length. A Kendall's tau-b correlation was run to determine the relationships between the dependent variables: A) proliferation and propagation, B) proliferation and shoot length, and C) shoot propagation and length (P < 0.001).

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