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A robust method for haploid sugar beet *in vitro* proliferation and hyperhydricity reduction

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ABSTRACT

Sugar beet is recalcitrant to *in vitro* tissue culture. Usually, proliferation of *in vitro* cultured rosette explants is a prerequisite for micropropagation. Although hormonal treatments can induce proliferation in sugar beet rosette explants, they may also result in some side effects. *In vitro* culture of sugar beet explants and some hormonal treatments make them more prone to hyperhydricity. Effects of media with different concentrations of 6-benzylaminopurine (BAP) and kinetin (Kin) on the proliferation and hyperhydricity of haploid sugar beet explants were investigated. It was observed that 0.2 mg L^{-1} Kin, with a reasonable amount of proliferation and minimum rate of hyperhydricity, performed better than BAP in different concentrations and combinations. The effect sizes of the treatments on the dependent variables were large. The correlation between proliferation and hyperhydricity of the treated explants was statistically negative and the association was large. However, the hormonal treatments without BAP or with the lowest amount of it produced the highest proliferation rate with the least hyperhydricity. The coefficient of determination was R^2 quadratic = 0.885. The results suggest that, in comparison with BAP, Kin is a potent plant growth regulator for the proliferation of sugar beet haploid explants that causes the least hyperhydricity. Although explants proliferated better in the presence of 0.01 mg L^{-1} BAP in combination with Kin than under Kin alone, the hyperhydricity of the proliferated explants decreased their suitability for *in vitro* propagation.

Key words: 6-benzylaminopurine, BAP, *Beta vulgaris*, cytokinin, doubled haploid, kinetin

Abbreviations:
CKs – cytokinins, DH – doubled haploid, H – haploid, Kin – kinetin

INTRODUCTION

Sugar beet is economically a very important plant (Řezbová et al. 2016). The sugar content of sugar beet has increased more than ten times after decades of breeding. The objectives of sugar beet breeding are: improving physiological (e.g. seed yield, germination, seedling vigour, biotic and abiotic

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stress resistances, root yield, bolting resistance, and monogermity), morphological (e.g. root shape), anatomical (e.g. cell size), and chemical (e.g. sucrose yield) characteristics (Bosemark 2006, Biancardi 2005). The sugar beet is normally an allogamous and biannual plant. Therefore, there is often a need to propagate the most favourable genotypes in order to multiply and preserve them for future breeding programmes or genomic analyses. In addition, an efficient tissue culture technique can be useful in biotechnological methods and molecular studies. Although clonal propagation of sugar beet *ex vitro* is possible, it is laborious and time-consuming. In spite of the fact that it is a species recalcitrant to *in vitro* growth and development (reviewed in Gürel et al. 2008), in comparison with *ex vitro* methods, its propagation *in vitro* is less demanding and much faster. *In vitro* proliferation of sugar beet precedes its propagation. In a closed culture vessel (*in vitro* conditions), hyperhydricity may adversely affect explants (Ivanova and van Staden 2010). In sugar beet *in vitro* tissue culture, hyperhydricity is a serious problem. The symptoms of hyperhydricity in sugar beet shoots include brittle, glassy, glossy, malformed leaves and a reduced number of leaves, accompanied by late rhizogenesis and poor acclimation to *ex vitro* conditions, which eventually may lead to tissue necrosis and explant death (Klimek-Chodacka and Baranski 2013).

Sugar beet explants are very sensitive to hyperhydricity while growing *in vitro* (Pazuki et al. 2017a). The problem can arise from an excess of the ammonium ion (NH_4^+) (Debergh et al. 1981), cytokinins (CKs) (Ivanova and van Staden 2011), high water potential or relative humidity of the *in vitro* medium (Debergh et al. 1981, Liu et al. 2017), ethylene production by explants (Kevers and Gaspar 1985), and stress-induced changes in the physiological state of explants (Kevers et al. 2004). Although the cause of hyperhydricity is not fully understood, it would seem that an efficient ventilation may be helpful (Ivanova and van Staden 2010), but, on the other hand, this may increase the risk of contamination.

In sugar beet tissue culture, the hormones from the CKs class are generally used to induce regeneration, proliferation, and propagation (reviewed in Gürel and Gürel 2013). 6-benzylaminopurine (BAP) is one of the growth regulators from among the CKs, which has been widely used in sugar beet ovule culture for gynogenesis (reviewed in Aflaki et al. 2017). However, the proliferated or regenerated plantlets can be hyperhydric (Tomita et al. 2013, Pazuki et al. 2017a). A hyperhydric sugar beet subculture on a medium with a new composition or under new conditions takes time and effort to produce a normal plant, which is a costly practice (Tomaszewska-Sowa 2012). The superior effect of kinetin (Kin) over BAP has been observed in sugar beet gynogenesis (Pazuki et al. 2017a, 2017b). It was recorded that BAP could cause higher hyperhydricity than Kin.

Many published studies report on the effects of various hormonal treatments on the regeneration, proliferation and propagation of different genotypes of sugar beet via diverse tissues (Mezei et al. 2006, Mishutkina and Gaponenko 2006, Gürel et al. 2011, Tomaszewska-Sowa 2012, Klimek-Chodacka and Baranski 2013, Tomita et al. 2013). However, in spite of the evidence for hyperhydricity provided by them (e.g. figures), none of them took the hyperhydric effect of applied treatments into account.

Haploid (H) material is generally a prerequisite for the production of doubled haploid (DH) plants. Producing DH plants, by providing full homozygosity after one generation, is very useful in the breeding of biennial plant species, for which the conventional methods take up to 10 years. Sugar beet DH lines may produce higher root yields than their initial lines (Kikindonov et al. 2016). Apart from the vital role of H material in DH plant production, H plants/explants *per se* are highly beneficial for forward and reverse genetics, cytogenetics, for inducing favourable mutations (e.g. resistance to biotic or abiotic stresses), gametosomatic hybridization, gametoclonal variation (Dwivedi et al. 2015), and protoplast fusion (Gürel et al. 2002). Production of H and DH plants in sugar beet has not been an easy task in comparison with other plant species (Aflaki et al. 2017). Considering the versatility and pivotal roles of H material, making it available for future studies is advantageous.

Sugar beet is not a species that is highly amenable to *in vitro* studies (Gürel et al. 2008, Aflaki et al. 2017). To propagate, it generally needs a fine-tuned concentration or combination of plant growth regulators. Micropropagation of sugar beet can be increased by applying CKs (Gürel and Gürel 2013). Since a side effect of CKs on sugar beet is hyperhydricity, and a hyperhydric shoot is difficult to be established as a normal plant (Liu et al. 2017), keeping the proliferation rate high and hyperhydricity low is rewarding. Moreover, identification of sugar beet genotypes with high proliferation potentials to be used in molecular breeding and improvement programmes often

requires screening of a large number of individual plants within sugar beet breeding lines (Ivic-Haymes and Smigocki 2005). Considering the difficulties such as hyperhydricity and genotype dependency, improving sugar beet proliferation is very crucial in the research and development of the crop. To our best knowledge, there is no publication on *in vitro* proliferation of sugar beet that takes the hyperhydricity of propagules into account. Therefore, we have examined ten different combinations of two types of CKs, i.e. Kin and BAP, in addition to three different amounts of Phytagel, to test the hypothesis of the favourable effect of Kin and the adverse effect of BAP on the proliferation and hyperhydricity of sugar beet H explants. The aim of the experiment was to find a treatment capable of inducing maximum proliferation while causing minimum hyperhydricity.

MATERIAL AND METHODS

Plant material and gynogenesis

Methods of producing gynogenic plant material had been explained previously (Pazuki et al. 2017a); here, the methods are described briefly. Inflorescences from a diploid self-fertile sugar beet (*Beta vulgaris*) genotype (i.e. SG2) bred at the Sugar Institute, Ankara, Turkey, were collected.

Gynogenic embryos were induced in three different media: one a control (GT0) and the other two with different concentrations of BAP (1 or 2 mg L^{-1}) (GT1 and GT2 in Tab. 1). Each treatment consisted of three Petri dishes as replicates (Fig. 1).

Pre-proliferation medium for gynogenic plantlets

The medium contained MS salts and vitamins (Murashige and Skoog 1962), 30 g L^{-1} sucrose, plus 0.5 mg L^{-1} BAP, solidified with 2.8 g L^{-1} Phytagel™, (HT1 medium, defined in Tab. 1). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 100 kPa above atmospheric pressure for 15 minutes. Each Magenta™ box was filled with 45 ± 5 ml of the autoclaved medium. Between 1 and 4 gynogenic plantlets were subcultured on the dishes, which were sealed using Parafilm®. The plantlets were propagated for two months and subcultured biweekly on the same medium (Fig. 1).

Nuclear DNA measurement

As described previously (Pazuki et al. 2017a), fresh leaf tissue (1.5 cm^2) of *in vitro* cultured gynogenic sugar beet and fresh leaf tissue (1 cm^2) of common vetch (*Vicia sativa*) (2C = 3.65 pg) were chopped up simultaneously with a sharp razor blade in 400 µL of extraction buffer of CyStain UV precise P (Partec, Münster, Germany). The suspended nuclei were incubated for 30 s and then the suspension was passed through a CellTrics® 30 µm filter into a test tube. Next, 1600 µL of 4',6-diamidino-2-phenylindole (DAPI) was added to each test tube and staining proceeded for a few minutes. A Partec CyFlow Space flow cytometer (Partec, Münster, Germany) and Windows™ based Partec FloMax® software were used to analyze the samples and the results. To evaluate the precision of the measurements, coefficients of variation (CV) were determined. For all the assessed cases, CV was below 5%, which supports the reliability of the flow cytometric analysis. To estimate the absolute value of DNA content (1C) for each sample, Doležel and Bartoš's (2005) formula was used: (G1 peak mean of *B. vulgaris* / G1 DNA content (2C) of *V. sativa*) × G1 peak of *V. sativa*.

Mitotic analysis

The method had been described previously (Pazuki et al. 2017a); briefly, young leaves of *in vitro* grown H plantlets were treated with a 2×10^{-3} *M* solution of 8-hydroxyquinoline dissolved in distilled water for 3 h at room temperature, then fixed in a freshly prepared 96% ethanol:hydrochloric acid solution (2:1, v/v) for 15 minutes. The fixed leaves were then rinsed with distilled water and 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 to squash it under a coverslip. To spread the cells, the coverslip was tapped gently a few times. The excess orcein solution was sucked

Table 1. Chemical composition of the treatments

Variables in media*	GT0	GT1	GT2	HT0	HT1	HT2	HT3	HT4	HT5	HT6	HT7	HT8	HT9
Sucrose (g L^{-1})	100	100	100	30	30	30	10	10	10	10	10	10	10
BAP** (mg L^{-1})	–	1	2	–	0.5	0.5	0.25	0.1	0.05	0.01	–	–	–
Kinetin (mg L^{-1})	–	–	–	–	–	0.5	0.5	0.5	0.5	0.5	0.5	0.2	0.2
Phytagel (g L^{-1})	2.8	2.8	2.8	2.8	2.8	3	3	3	3	3	3	3	6.5

*All the media contained full strength MS salts and vitamins (Murashige and Skoog 1962)
**BAP – 6-benzylaminopurine

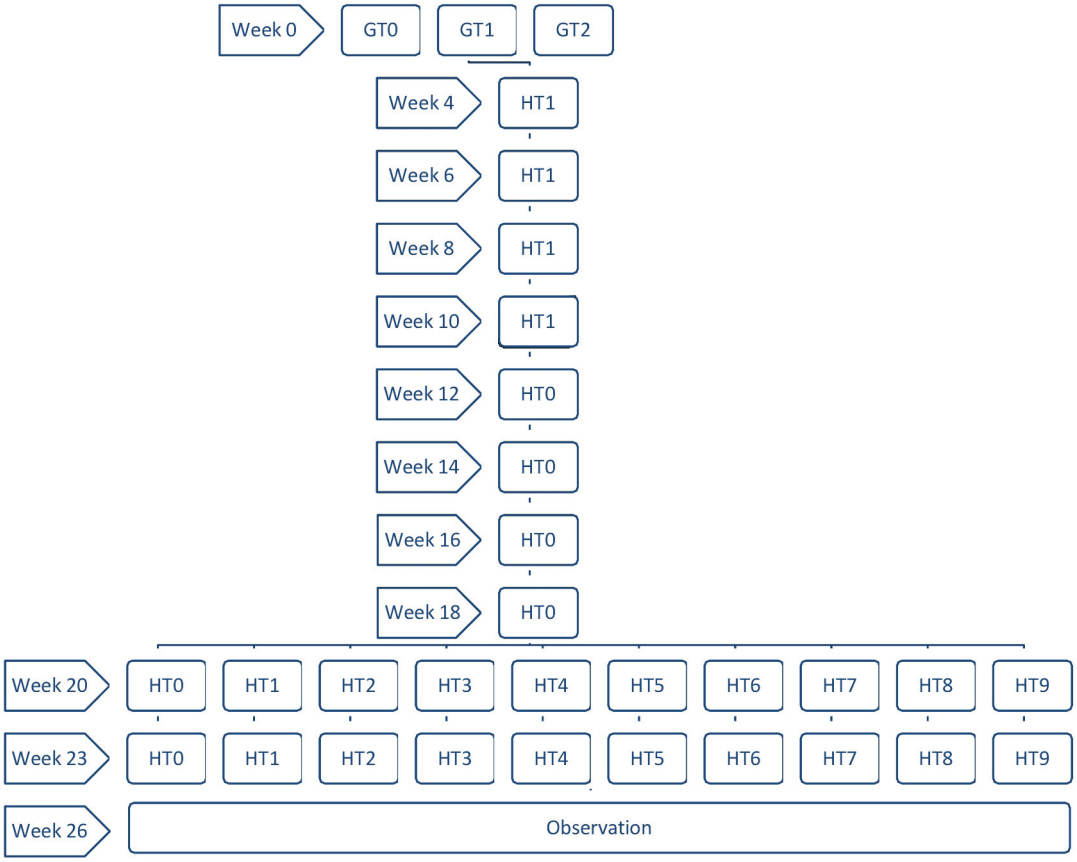

Figure 1. The sequence of hormonal treatments. The treatments are defined in Table 1. Sugar beet ovules were subjected to gynogenesis on an MS medium without hormones (GT0), or with 1 or 2 mg L^{-1} BAP (GT1 or GT2). The gynogenic plantlets were then propagated on HT1 medium (8 weeks). After that, they were subcultured biweekly for 8 weeks on a hormone-free (HT0) medium to minimize the effects of the previously applied treatments on the results of the subsequent treatments. Finally, after the initial propagation, the plantlets with three leaves were subcultured on ten defined media (Tab. 1) and after 3 weeks they were subcultured once more on the same media. Finally, the efficacy of the treatments on leaf proliferation was recorded after 6 weeks

up using a filter paper. After covering the coverslip with a filter paper, it was pressed with fingertips from side to side to spread metaphase plates. The chromosomes were counted under a light microscope.

Plantlet subculturing on a hormone-free medium

After two months, the plantlets were removed from the pre-proliferation medium, then they were propagated and subcultured on 45 ± 5 ml of a hormone-free medium (HT0) in Magenta™ boxes, being subcultured biweekly for two months to minimize the effects of the hormonal pretreatment (pre-proliferation medium) prior to subculturing on hormone-containing media (Tab. 1, Fig. 1).

Hormonal treatment

After two weeks of growing on the last medium (HT0), all the explants were propagated, randomly segregated, and each of them with three leaves to initiate was subcultured on the corresponding hormone-containing media. They were subcultured on 45 ± 5 ml of the media in a Magenta™ box. After three weeks, the explants were again subcultured on the same media. All the media contained MS salts and vitamins (Murashige and Skoog 1962), one or two types of hormone, sucrose, and solidifying agent (Tab. 1). After mixing all the constituents, except for the solidifying agent, the pH of the media was adjusted to 5.8, and then they were autoclaved at 121°C and 100 kPa above atmospheric pressure for 15 minutes.

Ambient conditions

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

Rooting and acclimation

The explants free from hyperhydricity symptoms were subcultured on HT9 to produce roots. After 25-35 days, the rooted explants were removed from the medium. They were potted in 1 L of sandy loam soil and covered with plastic bags to prevent dehydration. The bagged explants were put in a walk-in growth chamber to acclimate. The chamber provided an 18 h photoperiod and a constant temperature of 24 ± 2°C, and a relative humidity of 85 ± 10%. After 2 weeks, the plastic bags were gradually opened.

Observations and data analysis

After six weeks of growing on hormone-containing media, all the leaves grown from each explant were counted in order to calculate and analyze the effects of the treatments on proliferation. In addition, hyperhydricity of the explants resulting from each treatment was recorded in each replication. An explant was considered hyperhydric if at least one of its new leaves had developed symptoms of hyperhydricity. By recording these observations, quantitative and qualitative effects of the treatments could be analyzed (Fig. 1).

The experiment was carried out in a completely randomized design with ten hormonal treatments and four replicates. Each replicate consisted of 36 explants, making up 144 explants per treatment. A total of 1440 explants were cultured.

Observation records were tested for meeting the normality and homogeneity of variances using the Shapiro-Wilk and Lilliefors corrected Kolmogorov-Smirnov tests and *Levene's* test. The results of the treatment effects on proliferation met the normality assumption, but they did not meet the assumption of homogeneity of variances; therefore, they were analyzed using *Welch's* adjusted F ratio for one-way analysis of variance (ANOVA); for a post hoc analysis, the Games-Howell test was run ($p < 0.01$). For hyperhydricity results, the prerequisite assumptions were met. They were subjected to one-way ANOVA, and the means were compared using Tukey's honestly significant difference (HSD) at the 1% level of significance ($p < 0.01$) to test the significance of differences between groups. To estimate unbiased effect size (ES) of the independent variables, omega-squared and adjusted omega-squared values (ω^2 and *est.* ω^2) were computed (Cohen 1988, Field 2013). Since one of the prerequisite assumptions for Pearson's correlation coefficient (r) is a linear relationship between two variables, and Kendall's tau-b (τ_b) outperforms Spearman's rank-order correlation (r_s) asymptotically in terms of asymptotic relative efficiency (Croux and Catherine 2010, Xu et al. 2013), after removing outliers (HT0), a Kendall's tau-b (τ_b) correlation coefficient was computed to determine the relationship between proliferation and hyperhydricity, each of them with several tied ranks. A polynomial regression analysis was employed to fit the data with an appropriate model, i.e. a quadratic model. A Windows™-based SPSS® program (IBM Corp. Released 2015, IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp) was used for the statistical analyses and graph drawing. The result for proliferation was shown as the number of leaves per explant, and hyperhydricity was shown as a percentage. The results were expressed as proliferation, the percentage of hyperhydric regenerants, Kendall's tau-b (τ_b) correlation coefficient, and polynomial quadratic regression (Howell 2012).

RESULTS AND DISCUSSION

In this paper, we have reported on an efficient method of propagating sugar beet H explants through *in vitro* proliferation while minimizing hyperhydricity. The method has been efficiently used for many other H/DH genotypes, and wild/commercial *Beta* species (unpublished data). Here, the results for a haploid genotype are presented and their significance is discussed.

Flow cytometry analysis and chromosome counting using light microscopy confirmed a haploid set/number of chromosomes for all the plantlets. Relative fluorescence intensity was measured to estimate DNA content by flow cytometry using fresh young leaves of sugar beet. Histograms showed minimal amounts of background debris, G1 peaks were symmetrical and the variation was low. G1 DNA content of haploid explants was calculated using Doležel and Bartoš's (2005) formula [(122.07 / 502.16)] × 3.65 = 0.887 pg. In addition, using light microscopy, the recorded chromosome number of the gynogenic explants was 9 (n = 9). The records are in agreement with a previous cytological study (Weber et al. 2010).

Proliferation results showed that, in comparison with HT0 treatment, all the hormone-containing media were better in generating new leaves. However, ANOVA and post hoc analysis provided detailed data about their efficacy, which were statistically significantly different.

The results of proliferation (*Levene* F (9, 1430) = 22.434, $p < 0.001$) were examined statistically

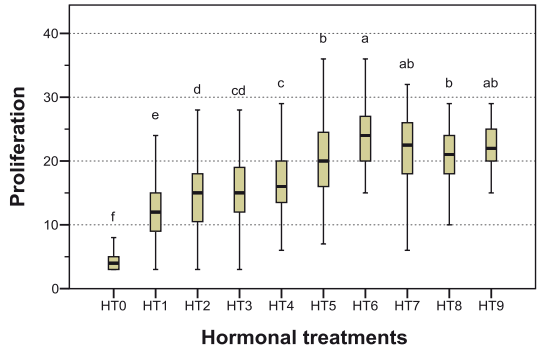

Figure 2. Effect of ten hormonal treatments on leaf proliferation (total number of leaves induced to grow from each explant) of sugar beet explants cultured *in vitro* with three leaves. The observations were made after six weeks of growing on the media defined in Table 1. The figure represents a comparison of means after analysis of variance (*Welch's* adjusted F ratio for one-way ANOVA) and Games-Howell post hoc test ($p < 0.01$). 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. The whiskers mark the largest and smallest observed values that are not statistical outliers

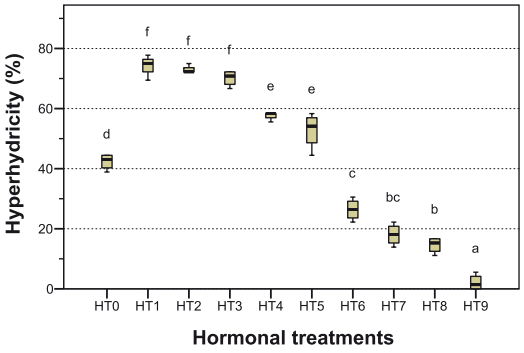

Figure 3. Effect of ten hormonal treatments on explant hyperhydricity (number of hyperhydric explants to the total number of treated explants) of sugar beet cultured *in vitro* with three leaves. The observations were made after six weeks of growing on the media defined in Table 1. The figure represents a comparison of means after analysis of variance (one-way ANOVA) and Tukey's honestly significant difference (HSD) test ($p < 0.01$). 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. The whiskers mark the largest and smallest observed values that are not statistical outliers

using *Welch's* adjusted *F* ratio (*Welch's F* (9, 566.215) = 902.567, $p < 0.001$). Games-Howell's post hoc test ($p < 0.01$) was done to compare the means (Fig. 2). The results of hyperhydricity (*Levene F* (9, 30) = 1.139, $p = 0.376$) were analyzed with one-way ANOVA (F (9, 30) = 263.978, $p < 0.001$). Then, a Tukey's a posteriori comparisons test ($p < 0.01$) was run to compare the means (Fig. 3).

The effect size of the hormonal treatments on sugar beet proliferation was *est.* $\omega^2 = 0.849$, whereas their effect on shoot hyperhydricity was $\omega^2 = 0.983$. The effect sizes of the treatments explained 84.9% and 98.3% of the total variances for proliferation and hyperhydricity, respectively. Based on Cohen's (1988) guidelines, both of the effect sizes are large. Thus, a majority of the improvements in the proliferation and hyperhydricity can be accounted for by the hormonal treatments.

A Games-Howell's post hoc test revealed that, in comparison with HT0 treatment, the treatment with 0.5 mg L^{-1} BAP (HT1) almost tripled the number of leaves (Fig. 2). However, this was at the expense of the total number of normally grown plantlets, because HT1 resulted in the highest rate of hyperhydricity (74.31%). Liu et al. (2017) had observed a similar effect of BAP on garlic (*Allium sativum* L). They reported that the proliferation coefficient increased in parallel with the increase in CKs concentrations.

The addition of Kin to the BAP-containing medium (HT2) increased the number of leaves in comparison with BAP alone (HT1). The number of leaves per explant increased incrementally when, respectively, 0.5, 0.25, or 0.1 mg L^{-1} BAP was accompanied by 0.5 mg L^{-1} Kin (HT2-4). Among the three combinations, HT4 almost quadrupled the number of leaves as compared with HT0 (15.23 and 4.22, respectively). Moreover, hyperhydricity of the proliferated explants decreased when BAP concentration was reduced (Fig. 3). Klimek-Chodacka and Baranski (2013) had reported that 0.3 mg L^{-1} BAP plus 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA) with 30 g L^{-1} sucrose caused blackening in two-thirds of *in vitro* propagated H explants. Liu et al. (2017) reported that higher levels of CKs increased hyperhydricity. Based on their experiment with garlic, in comparison with BAP, Kin caused higher hyperhydricity. In the present experiment, however, we observed that it was BAP that caused higher hyperhydricity than Kin in all the combinations and concentrations (Fig. 3).

The treatment with 0.2 and 0.5 mg L^{-1} Kin alone (HT8 and HT7, respectively), or the latter in combination with 0.05 mg L^{-1} BAP (HT5) increased the number of leaves nearly five-fold as compared with HT0 (Fig. 2). The effect of 0.5 mg L^{-1} Kin alone (HT7) on leaf proliferation was not statistically better than the effects observed for its

combination with 0.01 mg L^{-1} BAP or for 0.2 mg L^{-1} Kin alone (HT6 and HT8). The effects of the treatments on hyperhydricity were significantly different. HT7 resulted in 18.06% hyperhydric plantlets, while its combination with 0.05 mg L^{-1} BAP raised hyperhydricity to 52.78%. However, the 0.2 mg L^{-1} Kin-containing medium solidified with 3 g L^{-1} Phytagel (HT8) produced 14.59% hyperhydric plantlets (Fig. 3). In other pieces of research on sugar beet haploid gynogenesis from *in vitro* cultured ovules, BAP at 1 or 2 mg L^{-1} caused a statistically significant amount of hyperhydricity in gynogenic embryos (F (2, 41) = 7.102, p = 0.002) (Pazuki et al. 2017a), whereas Kin with a reasonable amount of regeneration did not result in hyperhydricity of the embryos (F (2, 106) = 22.05, $p < 0.001$) (Pazuki et al. 2017b).

The explants produced roots after approximately one month in HT9 medium (Tab. 1), after which they were potted and covered with plastic bags to acclimate. The survival rate of the acclimating plants was 98%. On an industrial scale, efficiency determines the most favourable protocol. A proliferated explant can produce more shoots, which is advantageous. However, for a given quantity of leaves the highest benefit can be obtained from higher quality leaves, which together defines efficiency. The highest percentage of leaf proliferation was recorded for the treatment with 0.5 mg L^{-1} Kin in combination with 0.01 mg L^{-1} BAP (HT6, 23.72%), which was followed by the treatment supplemented with 0.2 mg L^{-1} Kin and solidified with 6.5 g L^{-1} Phytagel (HT9 = 22.24%). However, the higher rate of hyperhydricity of the former resulted in lower efficacy. Therefore, HT9 was the most efficient treatment owing to its lowest rate of hyperhydricity (2.09%). This suggests that the higher concentration of the gelling agent (Phytagel) restricted water availability, ameliorated the effect of flooding stress, prevented water uptake by the explants, and therefore drastically decreased hyperhydricity among the plantlets. Klimek-Chodacka and Baranski (2013) had observed that hyperhydricity of H explants decreased the propagation rate by 32%. It was shown that Gelrite at 5 g L^{-1} resulted in a lower percentage of hyperhydric shoots than 2-3.5 g L^{-1} of it (Liu et al. 2017). On the other hand, in the present experiment, Kin at a low concentration (0.2 mg L^{-1}) induced a reasonable amount of proliferation from the explants while reducing at the same time the rate of hyperhydricity. In addition, it seemed that sucrose at 10 g L^{-1} was better than 30 g L^{-1}. Therefore, a lower concentration of sucrose could diminish costs without affecting propagation.

The medium of an *in vitro* tissue culture generally subjects explants to flooding stress, the main reason behind the hyperhydricity syndrome (Rojas-Martínez et al. 2010). Hyperhydric shoots are characterized by high water content. The water accumulates extensively in the apoplast of hyperhydric leaves (van den Dries et al. 2013). Water in the apoplast of plant leaves can hamper gas exchange by cells and cause hypoxia (Bailey-Serres et al. 2012), which probably brings about the symptoms of hyperhydricity (van den Dries et al. 2013) as a result of the generation of reactive oxygen species (Tian et al. 2017).

CKs can have an influential effect on the interaction between phytohormones. In *in vitro* culture, water vapour can be concentrated in a closed vessel and, as a result, relative humidity (RH) increases, which decreases transpiration by explants. The impaired transpiration can arise from hormone interactions and water vapour saturation, due to which the water vapour pressure gradient from plant to *in vitro* space will be minimal. Subsequently, the explant increases stomatal density (Carins Murphy et al. 2014), stomatal apertures (Arve et al. 2014), and abscisic acid (ABA) catabolism to mitigate the flooding stress (Arve et al. 2015). Despite the fact that in high relative humidity conditions the numbers of stomata and apertures increased, an *in vitro* cultured explant still could not get rid of excess water and alleviate flooding stress due to water vapour saturation. On the other hand, detached leaves of *Arabidopsis* developed under high relative humidity, although they produced more ABA, still suffered high water loss *ex vitro* (Arve et al. 2015), which could be because of the ABA's inability to close the stomata both *in vitro* and *ex vitro* (Arve et al. 2014). The negative effects of a closed chamber may be exacerbated by CKs. The shortcoming of ABA in closing the stomata could stem from the effects of CKs on stimulating stomatal opening and transpiration rate (Pospíšilová et al. 2000). Moreover, it has been indicated that CKs limited sucrose-induced ABA biosynthesis (Wojtania et al. 2015). Therefore, a high concentration of CKs affects explants by inhibiting ABA both *in vitro* and *ex vitro*, and limiting ABA synthesis. Ethylene is a flooding stress hormone (Kazan 2015). Silver ion (Ag^{+}) as an ethylene action inhibitor reversed hyperhydricity in *Dianthus chinensis* L. plantlets during *in vitro* culture (Gao et al. 2017). In the

presence of CKs, ethylene biosynthesis increased at higher sucrose concentrations (Wojtania et al. 2015). It has been suggested that hyperhydricity could possibly result from ethylene accumulation under high concentrations of CKs (Liu et al. 2017). We suggest that CKs evidently affect hyperhydricity.

By taking the interactions into account, it seems that CKs as a key factor in plant micropropagation and proliferation, and the gelling agent can be fine-tuned to boost the proliferation of explants and, at the same time, to minimize hyperhydricity. In the present experiment, the use of Kin at a very low concentration (0.2 mg L^{-1}), sucrose in the lower amount (10 g L^{-1}), and the solidifying agent at the higher concentration (6.5 g L^{-1} Phytagel) produced the highest proliferation with the lowest hyperhydricity. Almost all the explants treated with the HT9 medium were able to survive after acclimation to *ex vitro* conditions. The excellent survival of the explants might be due to the optimum concentration of Kin, lower concentration of sucrose, and higher amount of gelling agent, which might modulate the adverse effects of ABA and ethylene on plant growth and development.

From an economical point of view, higher rates of propagation with lower rates of hyperhydricity are very important. Although a few protocols are available for sugar beet propagation (reviewed in Gürel and Gürel 2013), none of them takes into account the deleterious effect of hyperhydricity and its correlation with the propagation rate. Many factors (e.g. gelling agent, carbohydrate source, ventilation and cultivar) can influence the rates of normally propagating explants. The percentage of regenerating explants in an experiment on H sugar beet explant propagation was as low as 10%. The blackening or necrosis of the explants led to the low efficiency of the explants developed in 0.3 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 30 g L^{-1} sucrose (Klimek-Chodacka and Baranski 2013). In the present paper, a correlation between hyperhydricity and proliferation rates has been reported for the first time for sugar beet *in vitro* culture. There was a statistically significant, negative correlation between the variables ($\tau_b = -0.648$, n = 36, $p < 0.001$). The coefficient of determination based on a quadratic model was $R^2 = 0.885$, F (2, 33) = 127.23, $p < 0.001$ (Fig. 4). The regression equation was: proliferation = 21.23 + 0.13 × hyperhydricity – 0.003 × hyperhydricity^2. Klimek-Chodacka and Baranski (2013) (based on Table 1 of the reference) had reported that the explants they used suffered from hyperhydricity and its consequent effect,

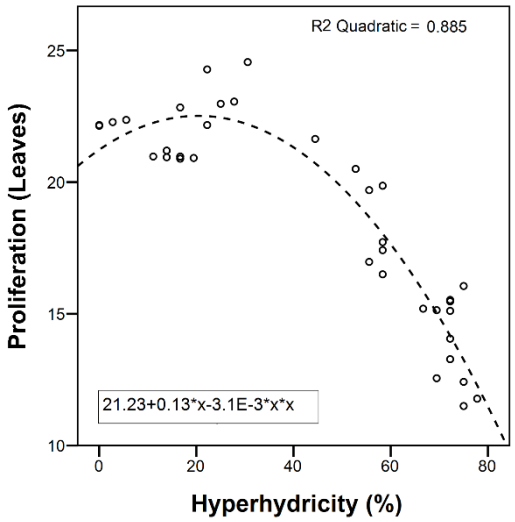

Figure 4. Correlation and regression between proliferation and hyperhydricity of haploid sugar beet explants grown *in vitro*. The two variables were negatively correlated ($\tau_b = -0.648$, n = 36, $p < 0.001$). The coefficient of determination: R^2 quadratic = 0.885, F (2, 33) = 127.23, $p < 0.001$. The regression equation: proliferation = 21.23 + 0.13 × hyperhydricity – 0.003 × hyperhydricity^2

necrosis. This is consistent with our findings; however we have provided an alternative medium to induce better proliferating explants.

To sum up, there are many factors to be considered for sugar beet proliferation and hyperhydricity. Among them, some are difficult to avoid (e.g. closed chamber of *in vitro* culture, which concentrates gases); however, the effects of some other ones can be eliminated or mitigated. As discussed, the concentrations of CKs, gelling agent, and sucrose are among the factors that can alter hyperhydricity. Changing these variables may help with successful tissue culture of sugar beet.

CONCLUSIONS

1. The present paper provides a comparative study on the proliferation of H sugar beet plantlets grown *in vitro* while alleviating the effect of hyperhydricity. In brief, our results indicated that Kin is a better plant growth regulator than BAP in proliferating non-hyperhydric plantlets.
2. To efficiently propagate sugar beet explants through proliferation with the least hyperhydricity, 0.2 mg L^{-1} Kin, 10 mg L^{-1} sucrose, and 6.5 mg L^{-1} Phytagel supplementing the MS medium (HT9) is recommended ($p < 0.01$).
3. Proliferation of explants increased by applying BAP and Kin in combination (HT6), but at the same time hyperhydricity of the explants was

exacerbated ($p < 0.01$). In contrast, although 0.2 mg L^{-1} Kin alone (HT9) did not induce the highest proliferation of explants, it caused the lowest rate of hyperhydricity ($p < 0.01$).

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AUTHOR CONTRIBUTIONS

A.P., F.A., S.G., E.G. and A.E. – designed and outlined the research; A.P. and F.A. – performed the experiment, analyzed and interpreted the data, and wrote the manuscript; S.G., E.G. and A.E. – edited it; A.P. and F.A. – contributed equally to this work.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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