BOLU ABANT IZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

GYNOGENESIS INDUCTION AND DOUBLED HAPLOID PLANT PRODUCTION IN SUGAR BEET (BETA VULGARIS)

DOCTOR OF PHILOSOPHY

ARMAN PAZUKI

BOLU, JANUARY 2019

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APPROVAL OF THE THESIS

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ABSTRACT

GYNOGENESIS INDUCTION AND DOUBLED HAPLOID PLANT PRODUCTION IN SUGAR BEET (BETA VULGARIS)
PHD THESIS
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BOLU, JANUARY 2019

The first chapter contains a brief introduction and a general literature review. Sugar was first extracted from sugar beet in 1747, with an approximate rate of 1.6% extractable sugar. During more than two centuries, researchers' attempts increased the sugar amount up to 20%. Majority of those successes were because of conventional breeding methods. In the early 19th century, a spontaneously induced haploid plant was discovered. The doubled haploid technique provides researchers with a complete homozygous plant, which is of high value for breeders, and let them shorten breeding duration for biennial plants from about ten years to two years. However, for sugar beet, from 1945 doubled haploid sugar beet technique was employed towards breeding sugar beets with other beneficial traits other than sugar concentration, i.e. resistance or tolerance to biotic or abiotic stresses. Several doubled haploid methods were examined, categorized *in vivo* and *in vitro* methods. Androgenesis, the most favorable methods mostly resulted in callus or diploid plants instead of haploid or doubled haploid plants. Except for gynogenesis, none of the applied methods was promising. Therefore, the only option was gynogenesis to be employed in sugar beet doubled haploid breeding programs. Sugar beet is an inter-breeding allogamous plant. Thus, there is a considerable variation among different varieties, cultivars, and genotypes. As a result, the available methods reported in the scientific literature sometimes cannot be successfully implemented for other genotypes. Sugar beet is an economically important crop for the countries in the temperate region of the northern hemisphere, including Turkey, which is a sugar beet seed importer country from western countries, e.g. Germany, the Netherland, Denmark, or Sweden. To produce sugar beet sustainably in Turkey, the country needs to breed and produce the required seeds and locally adaptable genotypes. Therefore, this project was launched to be a bridge between two parts of a long-term breeding program, started from more than a decade ago and is continued. The aims of these experiments reported in four chapters in the present thesis were haploid sugar beet induction through in vitro culture of unfertilized ovules (Chapter II); ameliorating hyperhydricity of the gynogenic explants (Chapter III); Doubled haploid sugar beet induction (Chapter IV); Increasing the propagation rate of the doubled haploid explants (Chapter V). The summaries of the chapters are provided in the following paragraphs.

The second chapter describes a protocol studying the effects of an interaction between cold pretreatment of six genotypes of sugar beet inflorescences at 4 °C for one week or more and 6-benzylaminopurine (BAP) concentrations (1 or 2 mg L^{-1}) to increase the response rate of haploid embryo induction.. Ovules were removed from the unfertilized flowers and cultured on in vitro media. The interaction of three variables was examined, i.e. genotype, cold pretreatment, and hormonal treatment. In comparison with freshly cultured ovules, cold pretreatment for one week almost doubled the mean of haploid plantlet induction rate. Supplementing BAP at 2 mg L^{-1} nearly doubled the induction rate of the cultured ovules, followed by 1 mg L^{-1} BAP in comparison with the hormone-free medium. Interaction of 2 mg L^{-1} BAP with one-week cold pretreatment induced the highest gynogenesis rate, but the hormonal treatment resulted in hyperhydricity. There was a considerable variation among the genotypes in their responses to the treatments. Genotype and cold pretreatment also showed a significant interaction. Cold pretreatment for longer than one week, i.e. 2–5 weeks resulted in similar or lower amounts of gynogenesis in comparison with the control (freshly cultured ovules). Ovules of one of the genotypes (SG3) treated with one-week cold pretreatment and 1 mg L^{-1} BAP produced the highest percentage of regenerants. BAP at 1 or 2 mg L^{-1} increased the gynogenesis rates 1.7 and 2 fold, respectively. Cold pretreatment increased the haploid embryo induction from a mean of 6.49% to 11.3% after one-week cold pretreatment.

The third chapter describes a study involving the effects of media with different concentrations of BAP and/or kinetin (Kin) as hormonal treatments, sucrose, and a solidifying agent (Phytagel) on haploid sugar beet explants' proliferation and hyperhydricity. After inducing haploid embryos and initial propagation of the explants, they were treated with ten different concentrations of the above-mentioned chemicals over six weeks. After applying the treatments, the mean of proliferation and the mean of hyperhydricity of the explants were compared. It was observed that Kin with a reasonable amount of proliferation and minimum rate of hyperhydricity performed better than BAP in different concentrations and combinations. Highest proliferation with the least hyperhydricity was obtained with 0.2 mg L^{-1} Kin, 10 g L^{-1} sucrose, and 6.5 mg L^{-1} Phytagel. The variables were negatively correlated ($\tau b = -.648$, $n = 36$, $p < .001$).

The fourth chapter describes a detailed study of a highly efficient protocol to multiply the number of haploid plants in sugar beet and subsequent chromosome doubling. In this chapter, the interactions between cold pretreatment, seven genotypes of sugar beet, and Kin to improve haploid embryo induction were studied. In addition, the effects of the color of ovules, flower bud position, and comma-form ovule on haploid embryo induction were investigated. Cold pretreatment for one-week, Kin supplementation, and genotype were influential in stimulating the ovules. Moreover, the main effects of flower bud position, ovule color, and comma-form ovule on gynogenic response were significant. Two-way and three-way interactions of the variables were also statistically significant. Kin at 0.05 or 0.5 significantly induced more gynogenic embryo induction in comparison with the control. The difference in gynogenic embryo induction between the most and the least responsive genotypes was about 4-fold. The effect of interaction between cold pretreatment and Kin was most prominent when 0.05 mg L^{-1} was used. However, for freshly cultured ovules, Kin was not statistically significant. The hormonal treatments' effects on the genotypes were different, e.g. a genotype (SG5) was highly benefitted from 0.5 mg L^{-1} Kin and

reached as much as 24% of induction, whereas another genotype (SG4) was not responsive to any of the hormonal treatments. When the effect of ovule position on inflorescence was studied, it was observed that the ovules removed from the flowers grew on the lower part of the inflorescence were more responsive and produced more gynogenic embryos than the ones removed from the upper part of the inflorescence. The ovules turned brown after one month produced higher percentages of the gynogenic embryo as compared with the ovules remained white. Colchicine at 5 g L^{-1} for 5 min was used to double the chromosome number of the haploid plantlets because the treatment over 3 or 7 min resulted in lower amounts of doubled haploid plants. However, the genotype responses to the doubling treatments were not significantly different.

In the fifth chapter, with an 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. The amino acid was supplemented to the most productive medium that is described in chapter III which contained 0.2 mg L^{-1} Kin, 10 g L^{-1} sucrose, and 6.5 mg L^{-1} Phytagel (i.e., treatment HT9). Proline at 0.3 mM induced the highest amount of proliferation and propagation, while proline-free medium resulted in the lowest amount of proliferation, and induced one of the lowest amounts of propagation. Proline at 0.3 mM induced the shortest shoots, whereas 0.1 mM proline induced the longest shoots. The proliferated explants were suitable for propagation (τb = 0.822, SE = 0.027, n = 75, $p < 0.001$). However, both proliferation and propagation showed negative correlation (τb = –0.565 and –0.601, SE = 0.061 and 0.054, respectively, n = 75, $p < 0.001$). For the first time, our results show beneficial effects of proline on *in vitro* proliferation and propagation of sugar beet.

The six chapter covers the main conclusions. In summary, it can be noted that haploid plantlet induction rate can be improved by cold pretreatment of inflorescences for one week at 4 °C. Moreover, BAP supplementation may induce more gynogenesis. However, the higher level of BAP may lead to higher abnormal development of emerged structures, e.g. hyperhydricity and necrosis. The technique appears highly genotype-dependent. However, Kin seemed a better alternative than BAP in inducing non-hyperhydric plantlets. Ovule color and the position of the flower bud on the inflorescence showed influential in gynogenesis, which was statistically significant. Proline at 0.4 mM might be deleterious to *in vitro* growth of sugar beet. Proline at 0.3 mM induced more proliferation. Although proline at 0.1 mM was less favorable, it yielded better proliferation and propagation rates in comparison with the 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.

KEYWORDS: Sugar beet, *Beta vulgaris*, Haploid, Doubled haploid, Ovule, Gynogenesis, Tissue culture, Propagation, Proline, Hyperhydricity, 6-Benzylaminopurine, BAP, Kinetin

ÖZET

ŞEKER PANCARINDA (BETA VULGARİS) GİNOGENEZİS İNDÜKSİYONU VE KATLANMIŞ HAPLOID BİTKİ ÜRETİMİ
DOKTORA TEZİ
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BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
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Tezin ilk bölümü kısa bir giriş ve genel bir literatür taramasını içermektedir. Yaklaşık %1.6 ekstrakte edilebilir bir şeker oranına sahip olan şeker pancarından şeker, ilk olarak 1747'de elde edilmiştir. İki yüzyıldan fazla bir süre boyunca, araştırmacıların girişimleri, bitkideki şeker oranını %20'ye kadar arttırmış, ve bu başarının büyük çoğunluğu ise geleneksel ıslah yöntemlerine atfedilmiştir. 19. yüzyılın başlarında, kendiliğinden oluşan ilk haploid şeker pancarı bitkisi keşfedilmiştir. Katlanmış haploid tekniği, yüksek değere sahip tam bir homozigot bitki sağlar ve iki yıllık bitkilerin ıslah sürelerini yaklaşık on yıldan iki yıla kadar kısaltır. Bununla birlikte, haploid bitki üretim tekniği 1945'ten itibaren, biyotik veya abiyotik streslere karşı direnç veya tolerans özellikleri gibi, şeker konsantrasyonu dışındaki diğer faydalı özelliklere sahip bitkilerin üretimi için yaygın olarak kullanılmıştır. Bir çok haploid üretim yöntemi denemiş olup, bunlar *in vivo* ve *in vitro* yöntemler olarak kategorize edilmişlerdir. En yaygın yöntem olan androgenezis çoğunlukla haploid veya katlanmış haploid bitki yerine daha çok kallus veya diploid bitkiler elde edilmesi ile sonuçlanmıştır. Ginogenezis dışında, uygulanan yöntemlerin hiçbiri umut verici olmamış, bu nedenle, şeker pancarı ıslah programlarında kullanılacak katlanmış haploid bitkilerin üretimi için tek seçenek ginojenez olmuştur. Şeker pancarı, kendine döllenen allogam bir bitkidir. Dolayısı ile, farklı çeşitler ve genotipler arasında önemli genetik bir varyasyon her zaman vardır. Bilimsel literatürde bildirilen mevcut yöntemler her şeker pancarı genotipi için başarıyla uygulanamayabilir. Şeker pancarı; Almanya, Hollanda, Danimarka veya İsveç batı ülkelerinden şeker pancarı tohum ithal eden Türkiye de dahil olmak üzere, kuzey yarımkürenin ılıman bölgelerinde bulunan tüm ülkeler için ekonomik olarak önemli bir üründür. Ülkemizde sürdürülebilir bir şeker pancarı üretimi yapabilmek için, ülkenin gerekli tohumları ve yerel olarak adapte edilmiş genotipleri ıslah etmesi ve üretmesi gerekir. Bu nedenle, bu tez çalışması, on yıldan daha uzun bir süre önce başlatılan ve hala devam eden iki parçalı bir ıslah programı arasında bir köprü olarak başlatılmıştır. Bu tez kapsamında, dört bölüm halinde sunulan deneylerin amacı, in vitro kültürü yoluyla döllenmemiş ovüllerden haploid şeker pancarı indüksiyonunu (Bölüm II); ginogenik eksplantların hiperhidrisitesinin iyileştirilmesini (Bölüm III); katlanmış haploid şeker pancarı üretimini (Bölüm IV); ve katlanmuş haploid eksplantların çoğaltım oranının arttırılmasını (Bölüm V) sağlamak olup, bu bölümler aşağıda özetlenmiştir.

İkinci bölümde, şeker pancarında haploid embriyo indüksiyonunu artırmaya yönelik olarak, çiçek salkımının bir hafta veya daha uzun süre 4 °C'de tutulması ile

farklı 6-benzilaminopurin (BAP) konsantrasyonları (1 veya 2 mg L^{-1}) arasındaki interaksiyonu inceleyen bir protokol tanımlanmıştır. Ovüller, döllenmemiş çiçeklerden izole edilmiş ve *in vitro* ortam üzerinde kültüre alınmışlardır. Genotip, soğuk ön işlemi ve hormon uygulamaları olmak müzere üç farklı değişken arasındaki etkileşim incelenmiştir. Taze kültüre alınmış ovüller ile karşılaştırıldığında, bir hafta boyunca soğuk ön uygulaması, haploid bitki indüksiyon oranını neredeyse iki katına çıkarmıştır. Hormon içermeyen besiyeri ile kıyaslandığında, 2 mg L^{-1} BAP takviyesi, kültüre alınmış ovüllerin indüksiyon oranını neredeyse ikiye katlamış, bunu 1 mg L^{-1} BAP takip etmiştir. En yüksek ginogenez oranını, 2 mg L^{-1} BAP'nin bir haftalık soğuk ön uyugulama ile kombinasyonu sağlamıştır fakat hormone uygulaması hiperhidrisiteye yol açmıştır. Genotipler arasında uygulamalara verdikleri tepkiler açısından önemli bir varyasyon gözlenmiştir. Genotip ve soğuk ön uygulama arasında da önemli bir etkileşim gözlenmiştir. Taze kültüre alınmış ovüller (yani kontrol) ile karşılaştırıldığında, bir haftadan daha uzun süreli (2-5 hafta) soğuk ön işlem uygulaması benzer veya daha düşük oranda ginogeneze yol açmıştır. Bir hafta soğuk ön işlemi ve 1 mg L^{-1} BAP uygulanmış genotiplerden birinin (SG3) ovülleri en yüksek oranda rejenerant üretmiştir. 1 veya 2 mg L^{-1} BAP içeren ortamlardaki ginogenez oranları, kontrole göre sırasıyla 1.7 ve 2 kat artış göstermiştir. Bir haftalık soğuk ön uygulaması, haploid embriyo indüksiyonu oranını %6.49'dan %11.3'e yükseltmiştir.

Üçüncü bölüm, farklı konsantrasyonlarda BAP veya kinetin (Kin) hormonları, sükroz ve bir katılaştırıcı madde olan Phytagel'i içeren ortamların haploid şeker pancarı eksplantlarının çoğaltımı ve hiperhidrisitesi üzerindeki etkilerini içeren çalışmaları tanımlamaktadır. Haploid embriyoların uyarılması ve ilk üretim sürecini takiben, eksplantlar altı hafta boyunca yukarıda belirtilen kimyasalların on farklı konsantrasyonunu içeren ortamlarda denenmiştir. Uygulamalardan sonra, eksplantların proliferasyon ve hiperhidrisite ortalamaları karşılaştırılmıştır. Kabul edilebilir oranda proliferasyon ve minimum düzeyde hiperhidrisiteye yol açan Kin'nin, farklı konsantrasyon ve kombinasyonlarda uygulanan BAP'den daha iyi performans gösterdiği gözlenmiştir. En az hiperhidrisite ve en yüksek proliferasyon, 0.2 mg L^{-1} Kin, 10 g L^{-1} sükroz ve 6.5 mg L^{-1} Phytagel uygulandığında elde edilmiş olup, değişkenler arasında negatif bir korelasyon ($\tau b = -.648$, $n = 36$, $p < .001$) gözlenmiştir.

Dördüncü bölümde, haploid şeker pancarı bitkilerinin sayısının artırılmasında ve ardından kromozom sayısının iki katına çıkarılmasında oldukça etkili olan bir protokolün detayları sunulmuştur. Bu bölümde, haploid embriyo indüksiyonunu artırmak amacıyla, soğuk ön uygulaması, yedi farklı şeker pancarı genotipi ve Kin arasındaki etkileşim incelenmiştir. Ayrıca, ovüllerin renginin, çiçek tomurcuğunun çiçek salkımı üzerindeki pozisyonunun ve virgül-şeklindeki ovülün haploid embriyo indüksiyonu üzerindeki etkileri araştırılmıştır. Bir hafta ön soğuk uygulaması, Kin takviyesi ve genotipler, ovüllerin uyarılmasında etkili olmuştur. Çiçek tomurcuk pozisyonu, ovül rengi ve virgül-şeklindeki ovülün ginogenik tepki üzerindeki etkileri anlamlı bulunmuştur. Değişkenlerin iki yönlü ve üç yönlü etkileşimlerinin de istatistiksel olarak anlamlı oldukları tespit edilmiştir. Kontrol ile karşılaştırıldığında, 0.05 veya 0.5 mg L^{-1} Kin, anlamlı şekilde daha fazla sayıda ginogenik embriyo indüksiyonu oluşturmuştur. Ginogenik embriyo indüksiyonunda en çok ve en az yanıt veren genotipler arasındaki fark yaklaşık 4 kat olmuştur. Soğuk ön uygulama ve Kin arasındaki etkileşimin etkisi 0.05 mg L^{-1} Kin kullanıldığında en belirgin şekilde gözlenmiştir. Bununla birlikte, yeni kültüre alınmış ovüler için Kin'nin etkisi istatistiksel olarak anlamlı bulunmamıştır. Hormonal uygulamaların genotipler

üzerindeki etkileri farklı olmuş, örneğin SG5 genotipi 0.5 mg L^{-1} Kin'de yüksek oranda indüksiyona (%24) ulaşmış fakat başka bir genotip (örneğin SG4), hormonal uygulamaların hiç birine cevap vermemiştir. Çiçek salkımı üzerindeki ovül pozisyonunun etkisi incelendiğinde, çiçek salkımının alt kısmı üzerinde büyüyen çiçeklerden çıkarılan yumurtların, üst kısmından çıkarılanlara göre daha verimli oldukları ve daha fazla ginogenik embriyo ürettikleri görülmüştür. Bir ay sonra kahverengiye dönen övüller, beyaz rengini koruyan ovüllerle karşılaştırıldığında daha yüksek oranlarda ginogenik embriyo ürettikleri gözlenmiştir. 3 veya 7 dakika uygulandığında düşük miktarlarda katlanmış haploid bitki ürettiğinden, bu oranı artırmak için 5 g L^{-1} kolşisin 5 dakika süreyle uygulanmıştır. Bununla birlikte, kromozom katlanması işleminde gözlenen genotipik varyasyon önemli bulunmamıştır.

Beşinci bölümde, katlanmış haploid bitki üretimini arttırmak amacıyla, beş prolin seviyesinin (0.0, 0.1, 0.2, 0.3 veya 0.4 mM) eksplantların proliferasyonu, çoğaltımı ve sürgün uzunluğu üzerindeki etkileri karşılaştırılmıştır. Prolin, 0.2 mg L^{-1} Kin, 10 g L^{-1} sakaroz ve 6.5 mg L^{-1} Phytagel içeren ve III. Bölümde en başarılı uygulama olarak belirlenen (HT9) ortamına ilave edilerek denenmiştir. 0.3 mM prolin uygulaması, en yüksek proliferasyona ve çoğaltıma, prolin içermeyen ortam ise en düşük proliferasyona ve en düşük çoğaltım oranlarından birine yol açmıştır. 0.3 mM prolin en kısa sürgün boyunu, 0.1 mM prolin ise en uzun sürgün boyunu yol oluşturmuştur. Prolifere olan eksplantlar, çoğaltım için oldukça uygun bulunmuşlardır (τb = 0.822, SE = 0.027, n = 75, $p < 0.001$). Bununla birlikte, hem proliferasyon hem de çoğaltım negatif bir korelasyon göstermiştir (sırasıyla, τb = –0.565 and –0.601, SE = 0.061 and 0.054). Sonuçlarımız prolinin şeker pancarının in vitro proliferasyonu ve çoğaltımı üzerindeki olumlu etkilerini ilk defa ortaya koymaktadır.

Altıncı bölüm genel sonuçları içermektedir. Özetle, haploid bitki indüksiyon oranının, 4 °C'de bir hafta boyunca çiçeklerin soğuk ön uygulamasıyla artırılabilir. Diğer taraftan, BAP takviyesi daha fazla ginogenezi indükleyebilir. Bununla birlikte, yüksek BAP dozları, gelişen yapılarda hiperhidrisite ve nekroz gibi anormal gelişmeleri tetikleyebilir. Tanımlanan yöntem oldukça genotipe bağımlı görünüyor. Kin, hiperhidrik olmayan bitkileri uyarmada BAP'dan daha iyi bir alternatif gibi görünmektedir. Ovule rengi ve çiçek tomurcuğunun çiçek salkımı üzerindeki yeri, ginogenezde istatistiksel olarak anlamlı bulunmuştur. 0.4 mM prolin, şeker pancarının in vitro büyümesine zararlı olmuş ama 0.3 mM'deki prolin daha fazla proliferasyon sağlamıştır. Her ne kadar 0.1 mM prolin daha az etkili olsa da, prolin içermeyen besiyerine kıyasla daha iyi proliferasyon ve çoğaltım oranlarının elde edilmesini sağlamıştır. En uzun sürgünler 0.1 mM prolin içeren ortamda elde edilirken, en kısa sürgünler 0.3 mM prolin içeren ortamda elde edilmiştir.

ANAHTAR KELİMELER: Şeker pancarı, *Beta vulgaris*, Haploid, Katlanmış haploid, Ovül, Ginogenezis, Doku kültürü, Çoğaltım, Prolin, Hiperhidrisite, 6-Benzilaminopürin, BAP, Kinetin

LIST OF PAPERS

This thesis is based on four published papers listed below:

I. Pazuki A, Aflaki F, Gürel E, Ergül A and Gürel S (2018a) "Gynogenesis induction in sugar beet (*Beta vulgaris*) improved by 6-benzylaminopurine (BAP) and synergized with cold pretreatment", Sugar Tech, 20: 69-77. doi: 10.1007/s12355-017-0522-x

II. Pazuki A, Aflaki F, Gurel E, Ergul A and Gurel S (2017) "A robust method for haploid sugar beet (*Beta vulgaris*) *in vitro* proliferation and hyperhydricity reduction", Folia Hort, 29: 241-250. doi: 10.1515/fhort-2017-0022

III. Pazuki A, Aflaki F, Gürel S, Ergül A and Gürel E (2018b) "Production of doubled haploids in sugar beet (*Beta vulgaris*): an efficient method by a multivariate experiment", Plant Cell Tiss Org Cult, 132: 85-97. doi: 10.1007/s11240-017-1313-5

IV. Pazuki A, Aflaki F, Gürel S, Ergül A And Gürel E (2018c) "The effects of proline on in vitro proliferation and propagation of doubled haploid sugar beet (*Beta vulgaris*)", Turkish Journal of Botany, 42: 280-288. doi: 10.3906/bot-1709-14

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LIST OF ABBREVIATIONS AND SYMBOLS

ABA : Abscisic acid

ANOVA : Analysis of variance

BAP : 6-Benzylaminopurine

CK : Cytokinin

DAPI : 4',6-diamidino-2-phenylindole

NAA : 1-Naphthaleneacetic acid

RH : Relative humidity

ROS : Reactive oxygen species

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CHAPTER I

1 Introduction

Sugar beet (*Beta vulgaris* ssp. *vulgaris*) is agriculturally an important crop, because it can accumulate about 20% of its root fresh weight as sugar. Among the 120 countries producing sugar, 70 of them produce it from sugar cane, 40 of them from sugar beet, and 10 of them produced sugar from both crops. European countries' sugar beet production comprises 68.2% of the world production. From 2014 to 2017, Turkey with an average of 18.4 million metric tonnes (MMT) production of sugar beet stands in fifth place after four European countries. During the same years, sugar beet production increased from about 17 to roughly 21 MMT, but the data suggests that it was in expense of increasing the harvest area (FAOSTAT, 2019).

Researchers suggest that sugar beet originated in areas around the Mediterranean region. Sugar beet is a halophyte species, and thus it is tolerant to salinity stress. It grows in different conditions, both as summer or winter crop. Commercial production of sugar beet is feasible in temperate regions of northern hemisphere (Francis, 2007).

B. vulgaris is a plant from Chenopodiaceae, a family of angiosperms commonly known as the amaranth family. It is a true diploid species with a haploid chromosome number of nine, a herbaceous, cross-fertilizing, dicotyledonous plant with annual and biennial genotypes. Cultivated sugar beet genotypes are, however, biennial. In its vegetative state, sugar beet is a rosette plant with a large taproot that is economically important for its sugar content. To reproduce, generally it needs a cold vernalization period followed by long days. The reproductive phase recognized with the emergence of an aerial stem with flowering branches. Sugar beet has perfect flowers with a tricarpellate pistil and five stamens and five narrow sepals. The ovary comprises a fruit that encloses a single seed. In multigerm seed, the ovaries of all

flowers are attached to a common receptacle. A receptacle with only a single flower produced monogerm seed (Figure 1.1) (Francis, 2007).

Two millennia ago, sugar beet was a garden vegetable, which was originated from various *Beta* species. In the 17th century, it was cultivated on a field, but as a fodder crop. Early in the eighteenth century, sugar beet was bred from a white root type of beet with a high concentration of sugar. However, development of beet into an industrial crop happened in the latter half of the century. For the first time in 1747, based on the roots' fresh weight, 1.6% sugar was extracted from sugar beet. Later on, further pieces of research were hindered due to the significant variation among beets, both in morphology and in sugar content. It took 55 years (1802) to triple the amount of sugar to 4% of the roots' fresh weight. During the upcoming years during the 19th century, the Napoleonic Wars, banning the importation of sugar of sugar cane, expanding sugar beet production area, developing and improving sugar-extracting factories, increasing the number of the factories, taxing cane sugar imports, and then taxing domestic beet sugar. All the changes happened during the first half of the 19th century, particularly the last one, encouraged the producers to develop new cultivars with higher sugar content and to improve the extraction efficiency. Because of the developments, the content of the sugar extracted in the middle of the 19th century reached 12%, then in 1880 culminated in 18–20%. The extracted sugar of sugar beet started in the mid-seventeenth century increased about 12-fold after 130 years of research and development. From that date until now, more than 130 years passed, but the change in the percentage of sugar in beet root' fresh weight is negligible (Francis, 2007).

Nonetheless, early in the 20th century, a spontaneously induced haploid plant of *Datura* was reported (Blakeslee et al. 1922). In 1945, a haploid sugar beet plant was for the first time produced. By taking advantage of haploid plants, researchers have been able to introduce new traits to the genotypes of sugar beet, e.g. tolerant and resistant to abiotic and biotic stresses.

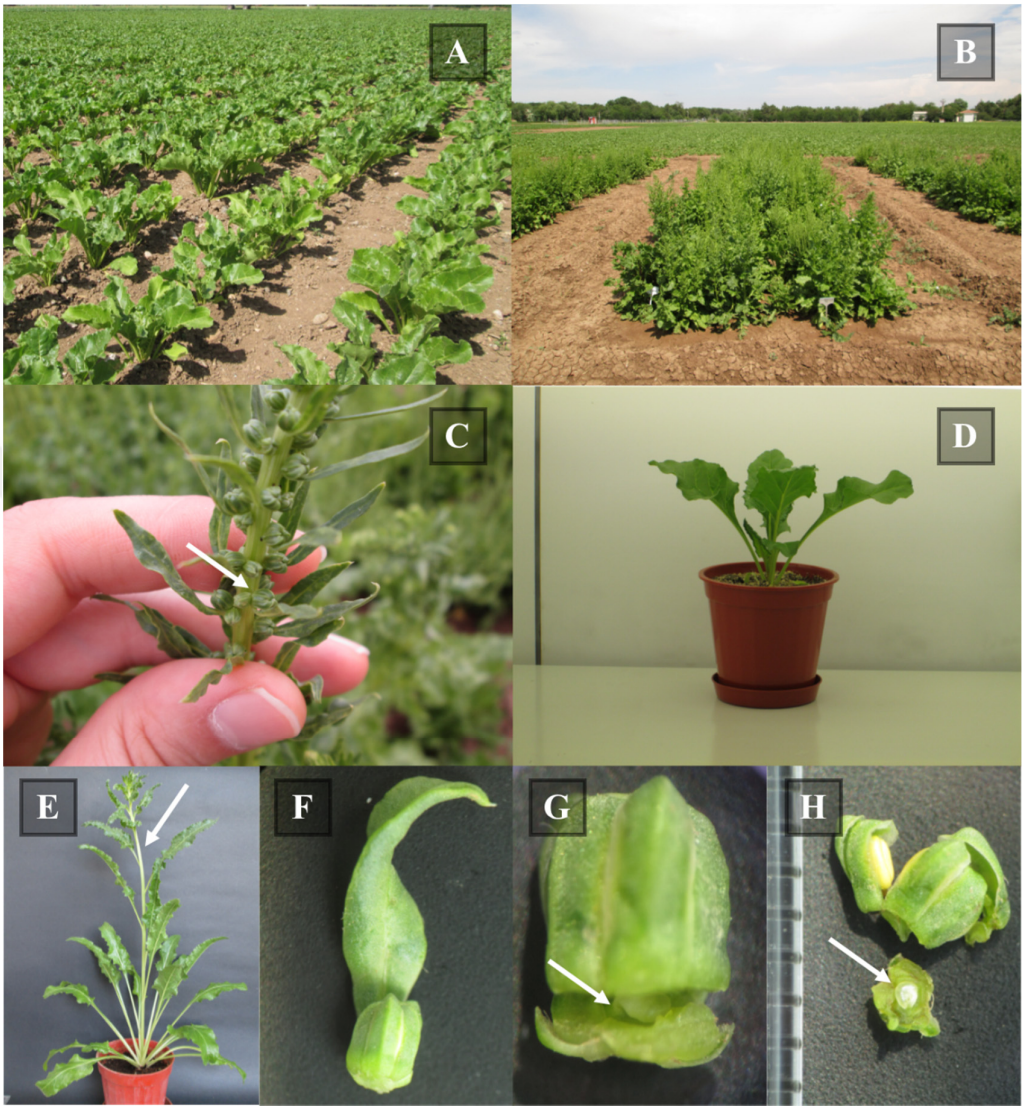

Figure 1.1. Sugar beet plant on the field, and its flowers. A) Sugar beet on field in rosette phase; B) A plot of the donor plants used for the experiments; C) A flowering stem of the donor plant, the white arrow indicates a multigerm (bigerm) flower; D) A potted sugar beet plant in rosette phase; E) A potted sugar beet plant in bolting phase, the white arrow indicates the bolted vertically growing stem; F) A monogerm flower of sugar beet detached from flowering stem; G) A monogerm flower of sugar beet dissected from ovary to reach the ovule, the white arrow indicates the ovule enclosed inside the ovary; H) A monogerm flower of sugar beet dissected from ovary to reach the ovule, the white arrow indicates the dissected ovule attached to the ovary's receptacle, and a ruler on the left indicates millimeters.

Haploid is a name referred to plants, diploid or polyploid, containing gametophytic chromosome numbers, i.e. one set of unpaired chromosomes (n). A haploid plant can be generated either spontaneously or artificially by different induction techniques. The latter can be either *in vivo* by parthenogenesis or *in vitro* by androgenesis (microspore and anther culture) and gynogenesis (ovule and ovary culture) (Forster and Thomas, 2005; Niu et al., 2014; Palmer and Keller, 2005; Murovec and Bohanec, 2012). Haploid plant production as a research tool has various beneficial applications ranging from plant breeding and genetic manipulation to plant genome/gene mapping (Niu et al., 2014). Its significant advantage is to achieve a complete homozygosity in a single generation (Niu et al., 2014). Instead, in conventional breeding, an acceptable level of homozygosity can be obtained after 6–7 generations followed by selections (De La Fuente et al., 2013). Of course, the time span can be doubled for biennial crops. Another remarkable advantage of haploid plants is the concurrent expression of recessive alleles masked in heterozygous condition (Doctrinal et al., 1989), which eases identification, evaluation and selection of useful traits (Klimek-Chodacka and Baranski, 2013).

World sugar production from sugar beet (*Beta vulgaris*) and sugar cane (*Saccharum officinarum*) with a ratio of 1.0:6.8 is around 216.79 Mt (FAOSTAT, 2018). Sugar cane yield is higher in tropical and subtropical regions, whereas sugar beet yields more in temperate regions, such as Turkey. Beet plant has been discovered about three millennia ago. Its breeding was started as early as 18th century in Germany. The breeding of the early plants increased sugar beet yield, which was further improved by using chemical fertilizers, agronomy and plowing machines (to read more, see Biancardi et al., 2010).

After the earliest discovery of haploid sugar beet (Levan, 1945), decades of attempts at producing haploid sugar beets have not yielded any applicable numbers of haploid plants or have repeatedly been thwarted by the formation of frequent non-haploids and callogenesis/rhizogenesis (reviewed in Gürel et al., 2008). The attempts consisted of *in vivo* and *in vitro* systems (see Figure 1 in Aflaki et al. 2017) e.g. i) natural polyembryony (Kruse, 1961); ii) inter-specific crossing (Cleij et al., 1968; Bosemark, 1971; Cleij et al., 1976); iii) inter-specific inter-ploidy crossing (Bosemark, 1971; Cleij et al., 1976); iv) conspecific inter-ploidy crossing (Bosemark, 1971; De

Jong and De Bock, 1978); v) pollinating with irradiated pollen (Bosemark, 1971), and by far the most favored technique, androgenesis (Banba and Tanabe, 1972; Welander, 1974; Rogozinska et al., 1977; Rogozinska and Goska, 1982; Van Geyt et al., 1985; Speckman et al., 1986). Up to the early 1980s, doubled haploid sugar beets were obtained following interspecific hybridization or using irradiated pollen, but the results were infrequent or in very small numbers. Moreover, the inability to develop haploid sugar beets through androgenesis convinced researchers to take advantage of gynogenesis.

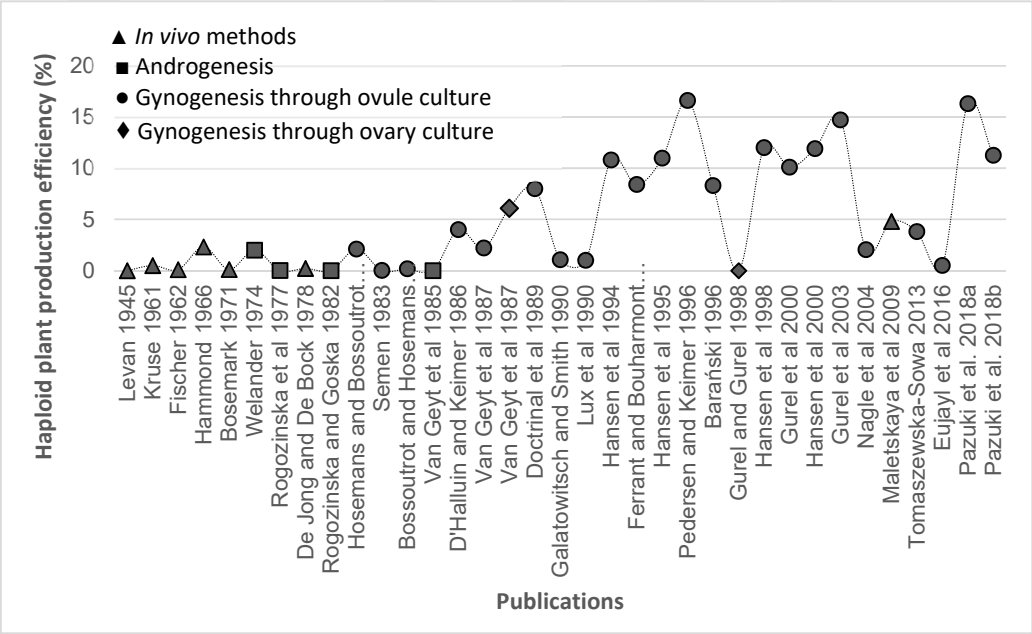

Figure 1.2. Attempts on improving haploid sugar beet production rate in a mean trend overview.

For sugar beet (*Beta vulgaris* L.), gynogenesis has been exploited for many years due to unresponsiveness to androgenesis. Despite the fact that studies on haploid plant production started as early as 1945, the gynogenesis rate for sugar beet is still less than 17% (Figure 1.2). A comprehensive study on sugar beet haploid and doubled haploid production can provide valuable knowledge for future research. The critical role of haploid and doubled haploid plant production in accelerating homozygosity and

the need for further improvement in gynogenesis rate are convincing reasons to focus on doubled haploid induction through unfertilized ovule culture.

Gynogenic haploid sugar beet induction using male-sterile genotypes was first reported by Hosemans and Bossoutrot (1983). They obtained 17 haploid plants out of 7237 unpollinated ovules cultured (0.23%), while the yield obtained by Seman (1983) was about 0.013%, which means 12 haploids amongst 93,125 plants observed after pollination. In 1985, Bossoutrot and Hosemans achieved 0.17% haploids from one hundred cultured ovules. Barocka et al. (1986) provided the first report on regeneration from callus of ovules in sugar beet. In 1990, Galatowitsch and Smith were able to produce five haploid calli out of 473 cultured ovules. Even though the *in vitro* culture of ovules from unfertilized closed flowers was more efficient as compared with classical techniques, until the late 1980s the average rate of haploids was only 1% (Lux et al., 1990). Doctrinal et al. (1989) obtained a rate of 6–10% plants per 100 cultured ovules, with an 81% haploidy rate. Based on the above findings, gynogenesis is the least favored method. However, for a species recalcitrant to androgenesis, e.g. sugar beet, it is a valuable technique. It could be presumed that gynogenesis is less efficient due to the lack of studies, which can be ascribed to the difficulties in the method including its intensive labor requirement. Instead, both simplicity and huge numbers of pollen are the main reasons that made androgenesis the most widely adopted method. It is quite likely that additional research focusing on gynogenesis will improve its efficiency.

For sugar beet doubled haploid production, polyembryony provided merely a few monoploids among thousands of screened plants (Kruse, 1961) and polyploidizing agent resulted in a large percentage of aneuploids (Bosemark, 1966), hence were not successful (Figure 1.2). It was later demonstrated that also the conventional methods of chromosome doubling done by applying colchicine on the main meristem were inefficient (D'Halluin and Keimer, 1986). This was in contrast to the results from a report indicating that using colchicine at initial stages of *in vitro* culture can have some advantages including: i) reduction in the amount of toxic material (colchicine, amyprophos methyl, trifluralin, etc.); ii) increase in the number of doubled haploid plants (Hansen et al., 1994); iii) costs reduction (Hansen et al., 1995).

Gynogenesis rate, also known as induced embryos from cultured unpollinated ovules, is affected by various environmental and genetic factors. Based on previous research on sugar beet, among the effective factors these ones can be considered: i) genotype; ii) growth condition; iii) position of flowers/ inflorescences on the stalk of donor plant; iv) development of ovules; v) pretreatment of flower buds; vi) composition of medium; vii) concentration of plant growth regulators; viii) temperature and seasonal conditions (reviewed in Aflaki et al., 2017).

Sugar beet breeding was started by selecting the plants with higher sugar content about the 17th century in today's Germany and Poland. This method was continuously used up to the mid-20th century in Germany, France, England, Russia, Poland, Denmark, Belgium, and Sweden. The use of the doubled haploid technique in sugar beet breeding was started in Sweden and developed in the other research centers in Europe and the USA. The newly introduced sugar beet genotypes are sold as seeds. Several of the major producers of elite seeds of sugar beet are also among the top five sugar beet producer countries, i.e. Russia, France, the USA, and Germany. Sugar beet was introduced to Turkey since the middle of 1920th. During the last years, Turkey has been the fifth largest sugar beet producing country after the above-mentioned countries. This is despite the fact that sugar beet seeds sown in Turkey are imported. Therefore, there has always been a need for producing sugar beet seeds in the country for long-term, sustainable production.

All sugar beet genotypes utilized as the mother plant in the breeding programs can be traced back to the plant bred in that time Prussia (modern time Poland) during the second half of 18th century. One of the techniques sugar beet seed producing companies is taking advantage of is doubled haploid sugar beet induction to introduced new traits to the developed genotypes. Sugar beet breeding has been completely benefitted from conventional breeding techniques during three centuries, hence after 1880, the concentration of sugar in root' fresh weight has not changed. Therefore, in the mid-nineteenth century, doubled haploid technique was introduced and continuously improved. The doubled haploid technique was employed in sugar beet breeding programs in parallel with conventional methods, e.g. mass selection, progeny selection and line breeding, inbreeding, recurrent selection, diploid or anisoploid synthetic varieties, hybrids, etc.

This project was launched in 2013 and financed by The Scientific and Technological Research Council of Turkey (TÜBİTAK) to produce and develop the first sugar beet genotypes within Turkish borders. The genotypes (all abbreviated as SG) employed in this project had previously been developed by Dr. Songül Gürel (coordinator of the above-mentioned TÜBİTAK project as well as co-supervisor of the thesis) of Sugar Institute (Ankara) via conventional methods of breeding. This project was developed as a PhD thesis to induce haploid and doubled haploid sugar beet from the developed genotypes, and to provide the required plant material that will be utilized for up-coming breeding steps.

1.1 Aims and Scope of the Study

In this study focusing on gynogenesis in sugar beet, besides providing elaborated methods for improvement of haploid and doubled haploid productions, we aimed to improve the success in propagation of obtained plants, the rates of which are still low or inefficient after years of laboratory tests. It has been attempted to study:

1) Haploid sugar beet induction through *in vitro* culture of unfertilized ovules;
2) Ameliorating hyperhydricity of the gynogenic explants;
3) Doubled haploid sugar beet induction;
4) Increasing the propagation rate of the doubled haploid plants.

CHAPTER II

2 Gynogenesis induction in sugar beet (*Beta vulgaris*): interaction of 6-benzylaminopurine (BAP) and cold pretreatment *(for more details, see paper 1, in the appendix)*

2.1 Introduction

Introducing a new variety requires establishing true breeding lines, through selfing for several generations, which is time-consuming. However, haploid and doubled haploid techniques provide homozygous lines during a considerably shorter time. Decades of attempts to produce haploid sugar beets have not been very efficient (reviewed in Aflaki et al., 2017). Sugar beet breeding is limited by the productivity of haploidization and genotype dependency of the applied methods, which may stem from its allogamous nature due to the self-incompatibility mechanism (Larsen, 1977). Sugar beet as a recalcitrant species to gynogenesis also is not very responsive to diploidization of haploid explants (Gürel et al., 2000; Hansen et al., 2000; Eujayl et al., 2016). Therefore, broadening the applicability of the old methods to other genotypes is generally welcomed. This research was aimed to compare the main effects of cold pretreatment and 6-benzylaminopurine (BAP), and to study their interaction effect on gynogenesis of sugar beet.

2.2 Materials and Methods

2.2.1 Plant material

Inflorescences of six diploid self-fertile sugar beet (*Beta vulgaris*) genotypes were studied. Apart from freshly used inflorescences, sugar beet inflorescences were kept in 4 °C in a refrigerator (Pazuki et al. 2018a).

2.2.2 Culture medium compositions and incubation conditions

Ovules of the genotypes were cultured either fresh or after one/two weeks of cold pretreatment at 4 °C. The ovules were cultured on a common medium, MS (Murashige and Skoog, 1962) salts and vitamins, 100 g L^{-1} sucrose, and 2.8 g L^{-1} phytagel™. As plant growth regulator, a control (hormone-free medium), and two different concentrations of BAP (1 or 2 mg L^{-1}) were compared. The Petri dishes containing ovules were kept in 16-h photoperiod, 24 ±2 °C, under irradiation of 35-µmol m^{-2} s^{-1}, and a relative humidity of 70 ±10%.

In addition, the effect of long-term cold (3, 4, and 5 weeks) pretreatment on the ovules was also investigated. Moreover, hyperhydricity, necrosis, and healthy plantlet rates as affected by the applied hormonal treatments were also studied. The regenerants were then subcultured on the same above-mentioned medium with a few modifications, i.e. sucrose 30 g L^{-1} plus 0.5 mg L^{-1} BAP to grow and develop (see Figure 1 in Pazuki et al. 2018a).

2.2.3 Flow cytometry analysis

Fresh leaf tissue from *in vitro* gynogenized sugar beet and fresh leaf tissue of common vetch (*Vicia sativa*) (as internal reference) were used for cytometric analysis. Nuclei were extracted using CyStain UV precise P (Partec, Münster, Germany), separated by CellTrics® 30 µm filter, and stained with 4',6-diamidino-2-phenylindole

(DAPI). A Partec CyFlow Space flow cytometer (Partec, Münster, Germany) (emission at 365 nm, and detection at 450 nm).

2.2.4 Chromosome counting

The chromosome number of the haploid and doubled haploid plantlets was counted under a light microscope after treating with 8-hydroxyquinoline (a 0.002 *M*, 3 h), then fixing in ethanol:hydrochloric acid solution (2:1 v/v, 15 min). A 3% orcein in 45% acetic acid was used for staining the metaphase chromosomes.

2.2.5 Experimental design and statistical analysis

In a completely randomized factorial design with three or six replicates, the percentages of ovules producing embryos, and the normal, hyperhydric, or necrotic gynogenic structures, and haploid plantlets induction were investigated after subjecting the results to analysis of variance (ANOVA), and appropriate post hoc analyses ($p \leq 0.05$) to determine the significance between groups.

2.3 Results

2.3.1 The effects of cold pretreatment, 6-benzylaminopurine (BAP), genotypic variation, and their two- and three-way interactions

The effects of one- and two-week cold pretreatment of the inflorescences, BAP supplementation, and genotypic variation on the rates of gynogenesis were statistically significant ($p < 0.01$) (Figure 2A, 2B, and 2C in Pazuki et al. 2018a).

The interaction of genotype × cold pretreatment was also significant (2D in Pazuki et al. 2018a), whereas, the interactions of genotype × hormonal treatment and cold pretreatment × hormonal treatment were not statistically significant. However, the three-way interaction was statistically significant ($p < 0.05$).

2.3.2 Effects of hormonal treatment on normality or hyperhydricity, and the effects of long-term cold pretreatment of inflorescences

The hormonal treatments resulted in varying amounts of normal or hyperhydric from the studied varieties (Figure 3A, 3B, and 3C in Pazuki et al. 2018a). The main effects of either long-term cold pretreatment or hormonal treatment were statistically significant, whereas, their interaction was not ($p < 0.05$).

2.3.3 The results of cytogenetic analysis

The flow cytometry analysis, aided by counting the chromosome number of haploid (1n = 1x = 9) and diploid (2n = 2x = 18) plants, confirmed that 82.9% of the generated plantlets were haploid (Figure 3D and Figure 4 in Pazuki et al. 2018a).

2.4 Discussion

Tissue culture condition is highly important for sugar beet doubled haploid plant production (Pedersen and Keimer, 1996). Certain stresses also may redirect normal gametophytic development to the sporophytic phase. The present chapter underlines the importance of cold pretreatment in combination with BAP to influence the percentage of gynogenesis from sugar beet ovules. Moreover, the results corroborated that genotype plays a major role in sugar beet gynogenesis (Doctrinal et al., 1989). Nevertheless, the applied method effects on increasing the average response of ovule are evident. The obtained result is one of the highest rates of embryo induction from sugar beet ovules as compared with previously published results (reviewed in Aflaki et al. 2017).

D'Halluin and Keimer (1986) observed that cold pretreatment for one week was not effective in changing the gynogenesis rate. Weich and Levall (2003) suggested the keeping the collected samples for one week at 8 ±2 °C is possible. The present experiment indicated the beneficial effect of cold pretreatment up to one week, whereas it indicated the detrimental effect of long-term cold pretreatment on gynogenesis rate.

Although D'Halluin and Keimer (1986) and Barański's (1996) did not observe any positive effects of BAP, others demonstrated that effectiveness of BAP treatment depends on genotype (Lux et al., 1990; Gürel et al., 2000; Tomaszewska-Sowa, 2012). The analyses suggest that BAP can have a significant effect on *in vitro* gynogenesis of sugar beet regardless and independent of cold pretreatment. However, since their interactions with the genotypes were statistically significant, it implies that their synergy depends on genotype.

Higher cytokinin concentrations result in hyperhydric to necrotic and/or malformed shoots (Doctrinal et al., 1989; Lux et al., 1990). In addition, induction of callus, dedifferentiation of some differentiated regenerants, and gynogenic embryos with abnormal morphology may also appear because of higher concentration of cytokinin. Therefore, several rounds of the subculture of the affected explants on lower levels of the hormone succeeded in redifferentiation, proliferation, and propagation of normally developing plants (Chapter III; Pazuki et al., 2017).

Other researchers observed that depending on the genotype, in fact, only a small fraction of gynogenic embryos are able to develop into viable plants (reviewed in Aflaki et al. 2017).

To overcome the genotype dependency of sugar beet in tissue culture systems is a continuing challenge (Gurel and Gurel, 2013; Pedersen and Keimer, 1996). Many have taken advantage of researching on highly responsive genotypes or doubled haploid plants (Pedersen and Keimer, 1996; Hansen et al., 1994; Hansen et al., 1995; Hansen et al., 1998; Hansen et al., 2000). Although the results obtained from the model or doubled haploid donor plants are among the bests, applying the same methods for other non-model, heterogeneous, or different genotypes usually cannot generate more or less similar expectation.

CHAPTER III

3 Haploid sugar beet (*Beta vulgaris*) *in vitro* proliferation and hyperhydricity reduction *(for more details, see paper 2, in the appendix)*

3.1 Introduction

Propagating the most favored genotypes of sugar beet, as an allogamous and biannual plant, in order to multiply and conserve them for future breeding programs or genomic analyses and biotechnological methods or molecular studies can be very advantageous. However, sugar beet explants experiencing *in vitro* tissue culture condition generally seriously suffer from hyperhydricity (Chapter II; Pazuki et al., 2018a).

6-Benzylaminopurine (BAP), a type of plant growth regulators from cytokinins (CK), has been frequently used in sugar beet gynogenesis (reviewed in Aflaki et al., 2017). However, the induced plantlets can show the symptoms of hyperhydricity (Tomita et al., 2013; Pazuki et al., 2018a; Chapter II), thus the recovery can take time and effort, be a costly practice (Tomaszewska-Sowa, 2012). The superior effect of Kin to BAP was observed in sugar beet gynogenesis (Chapter II and IV; Pazuki et al., 2018a, 2018b). It was observed that BAP resulted in the worse case of hyperhydricity than Kin did.

Although the effects of various plant growth regulators on sugar beet regeneration, proliferation and propagation were reported (Pazuki et al. 2017), none of them explicitly took hyperhydric side-effect of the applied treatments into account, notwithstanding the pieces of evidence for hyperhydric tissues provided in those publications (e.g. figures).

Sugar beet is a recalcitrant species to *in vitro* studies (Aflaki et al., 2017). Application of cytokinins can improve sugar beet micro-propagation (Gürel and Gürel, 2013). However, hyperhydricity needs to be addressed to keep the rates of proliferation high and hyperhydricity low. Numerous individual plants among breeding lines of sugar beet are required to find the genotypes with considerable proliferation potentials (Ivic-Haymes and Smigocki, 2005). For the first time, in the present experiment, hyperhydricity of the propagules as a side effect observed among proliferating sugar beet explants was studied to maximize proliferation and minimize hyperhydricity.

3.2 Materials and Methods

3.2.1 Plant material: gynogenesis and gynogenic plantlet pre-proliferation medium composition and condition

The methods followed to induce gynogenic plant material were briefly described previously (Chapter II; Pazuki et al., 2018a). Here, the methods are summarized briefly. A sugar beet (*B. vulgaris*) genotype response to *in vitro* condition was studied in the present experiment.

To induce gynogenic embryos similar media mentioned in the previous chapter were used. The medium composition was MS salts and vitamins, 30 g L^{-1} sucrose, and 0.5 mg L^{-1} BAP for pre-proliferation of the generated plantlets.

3.2.2 Nuclear DNA measurement and mitosis analysis

Cytometric analysis and chromosome number counting were done according to a previously published paper (Chapter II; Pazuki et al., 2018a).

3.2.3 Plantlet subculturing on a hormone-free medium and hormonal treatments' media

After two months in pre-proliferation medium, the plantlets were propagated and subcultured on a hormone-free medium for two months (Table 4.1, and Figure 1 in Pazuki et al. 2017). Then, the propagated explants were subcultured on the hormonal treatments to investigate the effects of the treatments on the proliferation and hyperhydricity of the explants (for more information read Pazuki et al. 2017).

Table 3.1. The treatments' chemical compositions.

Variables in media	HT	HT	HT	HT	HT	HT	HT	HT	HT	HT
Sucrose (g L^{-1})	30	30	30	10	10	10	10	10	10	10
BAP (mg L^{-1})	–	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 (gL^{-1})	2.8	2.8	3	3	3	3	3	3	3	6.5

3.2.4 Plantlet subculturing on a hormone-free medium, hormonal treatments, the ambient condition, rooting and acclimation

The condition *in vitro* explants grew was: 16-h photoperiod, 24 ±2 °C, under irradiation of cool white fluorescent tubes at 50 ±5 µmol m^{-2} s^{-1}, and relative humidity (RH) of 70 ±10%. The normally growing explants (without hyperhydricity symptoms) were subcultured on a rooting medium (HT9). After about one month, the rooted explants were removed from the *in vitro* medium, were potted, and covered with plastic bags to acclimate in 18-h photoperiod, 24 ±2 °C, and an RH of 85 ±10%.

3.2.5 Observation and data analysis

The explants were treated over six weeks on hormonal treatments. Then, the number of leaves grown from each explant were counted to evaluate the effects of the treatments on proliferation. Moreover, hyperhydricity of the explants was recorded if

at least one of the newly appeared leaves developed the symptoms (Figure 1 in Pazuki et al. 2017). For statistical analysis of the results, appropriate methods of analysis were used (for detailed information, see Pazuki et al. 2017).

3.3 Result

3.3.1 The results of cytogenetic analysis

Flow cytometry analysis and chromosome counting confirmed the haploid set of chromosome number for all the plantlets (read more in Pazuki et al. 2017).

3.3.2 The effect of hormonal treatments on proliferation and hyperhydricity

In comparison with the hormone-free medium (control), proliferation in the hormone-containing media was better in producing new leaves (Figure 2 in Pazuki et al. 2017). Supplementing 0.5 mg L^{-1} BAP almost tripled the number of leaves, but at the same time, it resulted in the highest rate of hyperhydricity. Combining Kin with BAP increased the number of leaves as compared with the medium with BAP alone. The result showed that the reduction in BAP concentration could mitigate hyperhydricity of the proliferated explants (Figure 3 in Pazuki et al. 2017).

3.3.3 The highest proliferation rate in the expense of inducing more hyperhydricity, and the correlation between them

Kin at 0.5 mg L^{-1} in combination with 0.01 mg L^{-1} BAP induced the highest number of leaf proliferation (M = 23.72, SD = 5.2). However, it resulted in a higher rate of hyperhydricity, hence the treatment was with lower efficacy. The medium with 10 g L^{-1} sucrose, 0.2 mg L^{-1} Kin, and a 6.5 g L^{-1} Phytagel was the most efficient treatment considering its lowest rate of hyperhydricity (2.09%), and on the other hand, since it induced a reasonable amount of proliferation.

3.4 Discussion

In this chapter, an efficient method for *in vitro* propagation of sugar beet haploid explants is reported. Klimek-Chodacka and Baranski (2013) observed that BAP at 0.3 mg L^{-1} combined with 1-naphthaleneacetic acid (NAA) at 0.1 mg L^{-1}, plus sucrose at 30 g L^{-1} caused blackening in two-thirds of the haploid explants. Others reported that CKs at higher amounts might worsen hyperhydricity (Liu et al. 2017). However, we observed that hyperhydricity is more severe in BAP supplemented media rather than that of Kin at any concentrations and with any combinations (Figure 3 in Pazuki et al. 2017). The results of the present experiment indicated that the gelling agent (Phytagel) at higher concentration could remarkably decrease hyperhydricity. In addition, the interactions and the cross-talks between RH, abscisic acid (ABA), ethylene, and CKs apparently affect hyperhydricity (Carins Murphy et al., 2014; (Arve et al., 2014; 2015; Pospíšilová et al., 2000; Wojtania et al., 2015; Kazan, 2015).

CHAPTER IV

4 *In vitro* doubled haploid sugar beet (*Beta vulgaris*) production *(for more details, see paper 3, in the appendix)*

4.1 Introduction

Breeding sugar beet through doubled haploid production has been continued over the last decades. Despite this, the plant is still recalcitrant to fully implementing this technique (reviewed in Aflaki et al., 2017). The allogamous nature and self-incompatibility of sugar beet (Larsen, 1977) increase genetic diversity. Thus, to utilize the doubled haploid production technique for sugar beet breeding, the tissue culture of the species needs to be the center of attention and attempts (Aflaki et al., 2017). In the present experiment, the effect of Kin in combination with one-week cold pretreatment was studied to achieve any improvement in the quality and quantity of the gynogenic plantlets. Moreover, for the first time, in the present experiment, the effect of the position of flower buds on the inflorescence was studied.

4.2 Materials and Methods

4.2.1 Plant material

Seven genotypes of sugar beet (*Beta vulgaris*) were studied in the present experiment. The detailed method and materials can be read in Pazuki et al. (2018b).

4.2.2 Culture medium composition, condition, and *in vitro* explantation

The media was MS (Murashige and Skoog, 1962) salts and vitamins, with 30 g L^{-1} sucrose, and solidified with 3 g L^{-1} gelrite™. The removed ovules were cultured on the Petri plates very similar to the method explained in the second chapter. The emerged plantlets were subcultured on a proliferation/propagation medium containing with 0.2 mg L^{-1} Kinetin (read more in Pazuki et al. 2018b).

4.2.3 Diploidization

An *in vitro* solidified culture medium was used to double chromosome set. After observing the effects of different concentrations of colchicine over varied time through several rounds of trials and errors, 5 g L^{-1} colchicine for 5 min was chosen and applied for doubling the chromosome number of the haploid plantlets (the details of the pre-experiments can be read in Pazuki et al. 2018b).

4.2.4 Flow cytometry analysis and chromosome counting

Flow cytometry analysis and chromosome counting were done based on the methods briefly described in chapter II (read more in Pazuki et al. 2018a and 2018b).

4.2.5 Observations and experimental design

One to two months after ovule culture initiation, the appeared gynogenic embryos were counted. Quantity and quality (hyperhydric and necrotic plantlet) of gynogenic embryos, and the effects of comma-form ovule and ovule color (white or brown) were investigated. In addition, doubling rates were also recorded. A completely randomized factorial design was implemented, and the obtained results were statistically analyzed using proper methods (the details of the analysis can be read in Pazuki et al. 2018b).

4.3 Results

Cold pretreatment significantly induced more gynogenesis. Higher concentrations of Kin generated more haploid embryos. However, the induction was genotype dependent. The interaction of Kin with cold pretreatment produced more gynogenic structures. The interactions between genotype and hormonal or cold treatments were statistically significant. The three-way interaction (genotype × cold pretreatment × hormonal treatment) showed statistically significant. More haploid embryos were recorded for the flower buds excised from the lower part of inflorescences. The ovules with brown color demonstrated more embryogenesis potential in comparison with the white ones. Comma-form ovules significantly generated a higher number of embryos. The explants were treated with 5 g L^{-1} colchicine over 5 min to double the chromosome number (Figure 4 in Pazuki et al. 2018b).

4.4 Discussion

Abiotic stress pretreatments may have stimulating effects on plant growth and development (Chen et al., 2011; Landi et al., 2016; Cardoso et al., 2016; Popova et al., 2016; Rout et al., 2016). Cold pretreatment sometimes showed ineffective in gynogenesis (Yang and Zhou 1982; D'Halluin and Keimer 1986). However, the same pretreatment could induce haploid embryogenesis (Lux et al., 1990; Svirshchevskaya and Dolezel, 2000; Gürel et al., 2000). Thus, it seems that this subject needs to be fully investigated. In the present experiment, cold pretreatment for one week was applied, because, as it was indicated in chapter II, the influential effect of cold pretreatment on sugar beet ovule gynogenesis was previously observed (Pazuki et al., 2018a).

Gynogenic embryos in sugar beet are not very high in quality and quantity (Pedersen and Keimer, 1996). The viable plantlets may be meager (0.5%) (Eujayl et al., 2016). However, by applying the hormonal treatments we were able to utilize beneficial effects of Kin at 0.5 mg L^{-1}, which almost doubled the rate of gynogenic embryo induction, without any symptoms of hyperhydricity or necrosis.

Taking into account the previous publication results, it was observed that the flower buds position on the branches might change gynogenesis efficiency (Doctrinal et al. 1989; D'Halluin and Keimer 1986), and considering that in our previous experiment (Chapter II; Pazuki et al., 2018a), we observed that even the position of the excised buds on the same branch had an effect on gynogenesis. For the first time, it was indicated that the ovules removed from the basal buds respond better and produce more gynogenic embryos. In addition, for the first time, it was indicated that ovule color might have an effect on gynogenesis rate; and the comma-form ovules, which once was merely mentioned (Van Geyt et al., 1987), the result of the present experiment indicated effective in changing haploid embryo induction.

The available reports on colchicine application suggest that it is more effective at higher concentrations (Hansen et al. 2000). In the present experiment, colchicine at a very high concentration over a very short time produced very effective and efficient results (to read more, see Pazuki et al. 2018b).

CHAPTER V

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

(for more details, see paper 4, in the appendix)

5.1 Introduction

Gynogenesis technique has not been efficient enough (Aflaki et al., 2017). This technique and other in vitro applied techniques may benefit from proliferating micro-propagules and propagating the resultants (Gürel et al., 2016). Although hormonal treatments, especially CKs can induce higher numbers of proliferation and propagation, they may also result in a few but serious side effects, e.g. abnormal growth, difficult rhizogenesis, callogenesis, necrosis, hyperhydricity, and inefficient acclimation (Pospíšilová et al., 2000; Klimek-Chodacka and Baranski, 2013; Górecka et al., 2017; Pazuki et al., 2018a).

Proline is a multifunctional amino acid (Szabados and Savoure, 2010) that plays roles responding to environmental stresses in many plant species (Franck et al., 2004; Dörffling et al., 2009; Manjili et al., 2012; Pazuki et al., 2015; Aksakal et al., 2017; Per et al., 2017).

CKs' effects on improving sugar beet *in vitro* propagation have been previously investigated (Chapter III; Pazuki et al., 2017). For the first time, here, the role of exogenously applied proline in *in vitro* proliferation and propagation has been studied (to know more, read Pazuki et al. 2018c).

5.2 Materials and methods

5.2.1 Plant material, gynogenesis medium composition, incubation conditions, and diploidization

A variety of sugar beet (*B. vulgaris*) genotype (SG3) was used in the present experiment. The methods were based on the previous chapters (Pazuki et al. 2018a and 2018b).

5.2.2 Proline treatment, and observation, experimental design, and statistical analysis

After propagating and randomly segregating doubled haploid plantlets, the explants were subcultured on five media: a proline-free media, plus four media supplemented with 0.1, 0.2, 0.3, or 0.4 mM proline. Three weeks after growing on the media, the number of propagated shoots and their length were recorded. The ambient condition and details of the method can be read in Pazuki et al. 2018c.

5.3 Results

5.3.1 The effect of proline on shoot proliferation, propagation, and length, and the correlations between them

The effects of proline treatments on mean rates of proliferation and propagation were statistically significant. The effect size of proline on the dependent variable was large enough to be taken into consideration for future research programs.

There was a very strong, positive, and significant correlation between proliferation and propagation (Figure 3A in Pazuki et al. 2018c). Between shoot proliferation and length, there was a moderate, negative, and significant correlation (Figure 3B in Pazuki et al. 2018c). The correlation between shoot propagation and

length was strong, negative, and significant (Figure 3C in Pazuki et al. 2018c). To read more about the results, see Pazuki et al. 2018c)

5.4 Discussion

The readers are recommended to see the original publication (Pazuki et al. 2018c) to read more about the discussion. Ivic-Haymes and Smigocki (2005)'s 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 culture of 8 sugar beet genotypes, including a model, highly regenerative tissue cultured clone, REL-1. Moreover, Ivic-Haymes and Smigocki (2005) reported that approximately 10% of the regenerants being 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 ±915 and 4.8 ±1.146, $p = 1.000$) after 3 weeks. In addition, all the explants were rooted after 5 ±2 weeks. Our observation under optimum *in vitro* conditions indicated that proline between 0.2 and 0.3 mM induced the highest rates of propagation. 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 in Pazuki et al. 2018c).

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 (Chapter III; 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 triggering by ROS signals (Verbruggen and Hermans, 2008).

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 observation, 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 mega doses (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 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.

CHAPTER VI

6 Conclusions

Haploid plantlet induction can be improved by cold pretreatment of inflorescences for one week at 4 °C. Moreover, BAP supplementation may induce more gynogenesis. However, the higher level of BAP may lead to higher abnormal development of emerged structures, e.g. hyperhydricity and necrosis. Cold pretreatment for more than one-week cannot be recommended, since gynogenesis rate plummets, particularly after the second week. The technique appears highly genotype-dependent. Two genotypes (SG1 and SG3) significantly produced more than other genotypes, whereas two other genotypes (SG2 and SG8) produced a very low amount of gynogenic embryos. An interaction between genotypes and cold pretreatments was observed. However, other two-way interactions were not statistically effective in changing the gynogenesis rates. The three-way interaction was indicated that might have an effect on the induction of gynogenesis. Therefore, to induce gynogenesis in sugar beet, pretreating the collected flowers with cold temperature (4 °C) and BAP is recommended. However, one is advised to consider the side effects of the higher concentration of BAP on the emerged plantlets.

On the other hand, the results of the experiment in the third chapter suggest that Kin is a better alternative than BAP in inducing non-hyperhydric plantlets. While BAP at any concentrations more or less (depending on the concentration) resulted in hyperhydricity, Kin at any concentration, particularly at 0.2 mg L^{-1}, induced a considerable amount of proliferation with a very low rate of hyperhydric plants. In addition, sucrose at lower concentration (10 g L^{-1}) in comparison with the generally used concentration (30 g L^{-1}) mitigated the hyperhydricity, but it did not decrease the proliferation rate. Solidifying agent (Phytagel) at higher concentrations (6.5 g L^{-1}) reduced hyperhydricity symptoms in comparison with lower concentrations (2.8 or 3 g L^{-1}).

The follow-up experiment, briefly described in the fourth chapter and fully explained in a published paper (Pazuki et al. 2018b), provides a method and the observed results that compare the efficiency of gynogenesis with the information provided in chapter II and a previously published paper (Pazuki et al. 2018a). Both chapters (II and IV) describing methods for gynogenic embryo induction confirm that cold pretreatment for one-week is effective. However, in the fourth chapter ovule color and the position of the flower bud on the inflorescence showed influential in gynogenesis, which was statistically significant. For experiment described in chapter IV, the observations from chapter III were taken into account, so that instead of BAP, Kin was used as the hormonal treatment, albeit at lower concentrations. Kin at 0.05 and 0.5 mg L^{-1} was statistically significantly better than hormone-free (control) treatment. Similar to the previously done experiment (chapter II, Pazuki et al. 2018a), genotypes responded differently to the treatments. All the two-way interactions were significantly influential in gynogenic embryo induction. Similarly, the three-way interaction of cold pretreatment × kinetin concentration × genotype statistically affected ovule responses. The flower buds taken from the lower part of an inflorescence showed more responsive to the treatments in comparison with the buds taken from the upper part of the inflorescence. By increasing the concentration of the antimitotic agent (colchicine) to 5 mg L^{-1} and treating the plants during a short time (5 min) the efficiency of chromosome doubling treatment was enhanced. Therefore, this method is suggested, i.e. increasing the concentration and treating over a shorter time.

In the sixth chapter and a published paper (Pazuki et al. 2018c), it was indicated that proline at 0.4 mM might be deleterious to *in vitro* growth of sugar beet. Proline at 0.3 mM induced more proliferation. Although proline at 0.1 mM was less favorable, it yielded better proliferation and propagation rates in comparison with the 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. A positive correlation between the proliferated explants and the propagation rates was observed. However, a negative correlation was observed between the shoot length and proliferation or propagation.

To understand the value of reached conclusions better, the readers are strongly recommended to read the four published papers based on the results of the experiments carried out by the Author. In the published papers, all the materials and methods are

elaborated. The obtained results are statistically analyzed, and the significance of the results are compared and discussed to interpret and describe the significance of the findings in light of what was already published in scientific literature and to explain new observations that were reported as results of the present study.

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APPENDICES

Published papers:

I. Pazuki A, Aflaki F, Gürel E, Ergül A and Gürel S (2018a) "Gynogenesis induction in sugar beet (*Beta vulgaris*) improved by 6-benzylaminopurine (BAP) and synergized with cold pretreatment", Sugar Tech, 20: 69-77. doi: 10.1007/s12355-017-0522-x

II. Pazuki A, Aflaki F, Gurel E, Ergul A and Gurel S (2017) "A robust method for haploid sugar beet (*Beta vulgaris*) in vitro proliferation and hyperhydricity reduction", Folia Hort, 29: 241-250. doi: 10.1515/fhort-2017-0022

III. Pazuki A, Aflaki F, Gürel S, Ergül A and Gürel E (2018b) "Production of doubled haploids in sugar beet (*Beta vulgaris*): an efficient method by a multivariate experiment", Plant Cell Tiss Org Cult, 132: 85-97. doi: 10.1007/s11240-017-1313-5

IV. Pazuki A, Aflaki F, Gürel S, Ergül A And Gürel E (2018c) "The effects of proline on in vitro proliferation and propagation of doubled haploid sugar beet (*Beta vulgaris*)", Turkish Journal of Botany, 42: 280-288. doi: 10.3906/bot-1709-14

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RESEARCH ARTICLE

Gynogenesis Induction in Sugar Beet (*Beta vulgaris*) Improved by 6-Benzylaminopurine (BAP) and Synergized with Cold Pretreatment

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Abstract For sugar beet (*Beta vulgaris*) breeding, producing homozygous lines through haploid and doubled haploid techniques are preferred over conventional and time-consuming methods. Doubled haploid sugar beet production necessitates inducing ovules to develop into haploid plants, referred to as gynogenesis. The protocol involves an interaction between cold pretreatment of six genotypes of sugar beet inflorescences at 4 °C for 1 week or more and 6-benzylaminopurine (BAP) concentrations (1 or 2 mg L^{-1}) to increase the response rate of haploid embryo induction. Compared with freshly cultured ovules (6.49%), cold pretreatment for 1 week almost doubled the mean of haploid plantlet induction rate (11.3%), whereas pretreatment for more than 1 week was not as effective as the control. Addition of 2 mg L^{-1} BAP to the culture medium nearly doubled the induction rate of the cultured ovules (10.75%), followed by 1 mg L^{-1} BAP (7.78%) in comparison with hormone-free medium (5.69%). The highest gynogenesis rate (37.8%) was achieved when ovules were cultured on medium containing 2 mg L^{-1} BAP following 1-week cold pretreatment. This combination approximately tripled the mean total haploid embryo induction rate of all the genotypes to 16.3% in comparison with the control (5.74%). However, the addition of BAP resulted in vitrification proportionately. As a result, 2 mg L^{-1} BAP decreased the normal plantlet emergence (NPE) to one-third (7.59%) while 1 mg L^{-1} BAP had a moderate effect (NPE: 18.98%) in comparison with hormone-free treatment (NPE: 24.35%). The results indicate that the combination of cold pretreatment and BAP is very effective in inducing haploid plants from recalcitrant genotypes of sugar beet, but BAP can have both advantages and disadvantages.

Keywords Sugar beet · Haploid · Cold pretreatment · *Beta vulgaris* · 6-benzylaminopurine (BAP)

Introduction

Introducing a new commercial variety requires establishing true breeding lines. Inbred lines in sugar beet have been conventionally produced through selfing for several generations or by successive full or half-sib crosses. Despite being relatively efficient, these methods are time-consuming and the lines are still relatively heterogeneous. In contrast, haploid and doubled haploid techniques produce homozygous lines within considerably shorter time.

Decades of attempts to produce haploid sugar beets since the earliest encounter with the first haploid sugar beet (Levan 1945) to the recent efforts using in vivo and in vitro systems have not yielded any applicable number of haploid plants or have failed by the formation of non-haploids, callogenesis, or rhizogenesis with high frequencies (reviewed in Gürel et al. 2008).

For sugar beet, however, two major bottlenecks limiting the applicability of haploidization are low frequency and genotype dependency of methods in their responses. The discrepancy in gynogenesis rates is evident even among

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ovules harvested from different branches of the same plant (D'Halluin and Keimer 1986), let alone between genotypes (Gürel et al. 2000). This inconsistency in the embryogenesis rate of a pool of ovules to a given treatment has made sugar beet a recalcitrant example for gynogenesis. Low or variable yield of sugar beet haploid and doubled haploid production stems from its allogamous nature owing to the self-incompatibility mechanism (Larsen 1977), which accounts for inbreeding depression due to the extreme types of selfing, haploidization and double haploidization (Szovenyi et al. 2014).

Although sugar beet is known as a recalcitrant species to gynogenesis, its genome doubling method has been claimed to be efficient (Weich and Levall 2003). This is in spite of the fact that other researchers had faced difficulties in diploidization of haploid explants and had not found it an efficient method for sugar beet (Gürel et al. 2000; Hansen et al. 2000; Eujayl et al. 2016). Haploid sugar beet production involves a two-step process: haploid gynogenic embryo induction and haploid plantlet regeneration. The first step may evolve from either embryogenesis directly (Weich and Levall 2003) or embryonic callus indirectly (Galatowitsch and Smith 1990).

An efficient and relatively easy method to induce haploids from as many genotypes as possible will be beneficial for sugar beet breeding. Researchers have proposed many factors contributing to the success rate of gynogenesis in sugar beet in terms of both quality and quantity, among which genotype (D'Halluin and Keimer 1986; Doctrinal et al. 1989; Lux et al. 1990; Weich and Levall 2003), stage of gamete development (Bossoutrot and Hosemans 1985), pretreatment of flower buds (D'Halluin and Keimer 1986; Lux et al. 1990; Gürel et al. 2000, 2003a), and in vitro culture media composition and condition have been evaluated (Doctrinal et al. 1989; Lux et al. 1990; Gürel et al. 2000; Weich and Levall 2003). Virtually all the highly reproducible and actual protocols routinely used or sponsored by breeding companies to produce haploid plants are unavailable or are not presented in detail to maintain their competitiveness in the global market (Pedersen and Keimer 1996).

Therefore, upgrading and updating the old methods to broaden their applicability to other genotypes, to move toward and actualize a genotype-independent method, is generally welcomed.

The objectives of this research were to compare the main effects of cold pretreatment and 6-benzylaminopurine (BAP) and their interaction effect on sugar beet gynogenesis and to study the limits of the stimulating factors, their effects on quantity and quality of gynogenized structures, and possibility of pushing back the boundaries of sugar beet recalcitrancy to gynogenesis.

Materials and Methods

Plant Material

Inflorescences (8–12 cm in length) of six diploid self-fertile sugar beet (*Beta vulgaris*) genotypes (SG1, SG2, SG3, SG4, SG6, SG8) bred at the Sugar Institute (Ankara, Turkey) were collected in the second half of June. The collected inflorescences were used either fresh or after storage at 4 °C in a refrigerator (i.e., cold pretreatment for 1, 2, 3, 4, and 5 weeks). Then 10–12 of the branches (after removing the bracts) were sterilized with sodium hypochlorite solution (6–14% active chlorine) (NaOCl) diluted in distilled water (DW) (23 mL NaOCl + 77 mL DW) plus Tween-20 (2 drops per 100 mL of the solution). After 30 min of manual shaking, the explants were rinsed with DW three times (a flow chart of the process is given in Fig. 1).

Culture Medium Compositions and Incubation Conditions

Ovules from all the genotypes were cultured either fresh or after 1 or 2 weeks of cold pretreatment at 4 °C. The ovules were removed one by one from ovaries under a stereomicroscope using forceps and a scalpel, and cultured on 90 × 15 mm disposable plastic Petri dishes containing 25 mL of autoclaved solid induction media. The common composition of the media was MS (Murashige and Skoog 1962) salts and vitamins, 100 g L^{-1} sucrose, and 2.8 g L^{-1} Phytagel™. As plant regulator treatments, in addition to the control (hormone-free medium), two different concentrations of BAP (1 or 2 mg L^{-1}) were used. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. Each treatment consisted of three replicates of Petri dishes and fifteen ovules per Petri dish (i.e., 45 explants per treatment). The dishes containing ovules were sealed with parafilm and were kept in a growth chamber with a 16-h photoperiod at constant temperature of 24 ± 2 °C, under irradiation of 35 µmol m^{-2} s^{-1} radiated by cool white light-emitting diode (LED) lamp (Shenzhen Modern Lighting Co., Ltd., Shenzhen, China) at a relative humidity of 70 ± 10%.

To evaluate the effect of long-term cold pretreatment, the ovules from all the genotypes were cultured after treatment for 3, 4, and 5 weeks under the same above-mentioned conditions in six replicates.

The emerged plantlets were then cultured on a medium containing MS salts and vitamins (Murashige and Skoog 1962), sucrose 30 g L^{-1}, Phytagel™ 2.8 g L^{-1}, plus 0.5 mg L^{-1} BAP to follow their growth and development into potted plants.

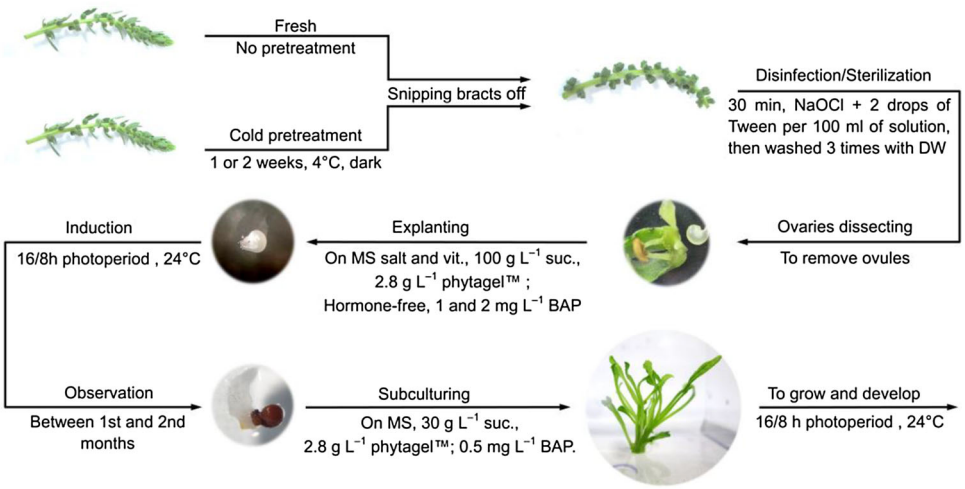

Fig. 1 Flow chart for the in vitro sugar beet gynogenesis protocol employed

In addition to the study of the effects of hormonal treatment on the quantity of gynogenesis, their effects on the quality of gynogenized structures were studied. The qualitative observations were vitrification, necrosis, and healthy plantlet rates affected by the three aforementioned hormonal treatments regardless of the cold pretreatments.

Flow Cytometry Analysis

Approximately 1.5 cm^2 of fresh leaf tissue from each in vitro cultured explant of gynogenized sugar beet having more than three leaves and 1 cm^2 of fresh leaf tissue of common vetch (*Vicia sativa*) (internal reference standard with $2C = 3.65$ pg) were all together chopped up with a sharp razor blade in a plastic Petri dish containing 400 µL of extraction buffer of CyStain UV precise P (Partec, Münster, Germany). The nuclei suspension of the chopped tissues was incubated for 30 s and then each suspension was passed through a CellTrics® 30 µm filter into a glass tube. Next, 1600 µL of 4′,6-diamidino-2-phenylindole (DAPI), the staining buffer, was added to each glass tube and staining proceeded for a few minutes at room temperature. Then they were kept in a dark and cold (4 °C) place until analysis. The samples were analyzed using a Partec CyFlow Space flow cytometer (Partec, Münster, Germany) equipped with a high-power UV LED fluorescence excitation laser at 365 nm to detect fluorescence emission at 450 nm. Windows™ based Partec FloMax® software was used for the analysis of results. For each sample, the mean coefficient of variation (CV) values was less than 5% for all the samples.

Chromosome Counting

To count the chromosome numbers of the produced plantlets, young leaves of haploid in vitro plantlets having more than three leaves or very young leaves of growth-chamber-grown diploid plants were treated with 8-hydroxyquinoline (a 0.002 *M* solution prepared with distilled water warmed to 60 °C for 15 min to dissolve all of the substance) for 3 h at room temperature, followed by fixation in a freshly prepared 96% ethanol/hydrochloric acid solution (2:1 v/v) for 15 min. Then the leaves were rinsed with and kept in distilled water. Next, a small excised piece of the leaf tissue was transferred to a drop of 3% orcein in 45% acetic acid on a slide. A coverslip was put on and gently pressed to squash the tissue. The coverslip was tapped gently a few times to spread the cells. Filter paper was used to suck up the excess orcein solution. To spread the metaphase plates further, the coverslip was covered with filter paper and then was pressed by fingertip from one side to the other. Finally, the chromosomes were counted under a light microscope.

Experimental Design

The experiment was carried out in a completely randomized factorial design (i.e., six genotypes × six pretreatment duration × three hormonal treatments = 108 treatments) with three or six replicates. Each replicate consisted of 15 ovules, making 45 or 90 explants per treatment. The results were expressed as the percentage of ovules producing embryos; the percentage of normal, vitrified, or necrotic gynogenic structures; and the percentage of haploid plantlets induced from the six genotypes.

Statistical Analysis

The rate of gynogenic embryo induction was recorded 1–2 months after ovule culture initiation. The data were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test at 5% level of

significance ($p \leq 0.05$) to determine the significance between groups. Windows™ based SPSS® (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

Results

Varying rates of gynogenesis were obtained from fresh and 1- and 2-week cold pretreated inflorescences at 4 °C [$F(2,108) = 8.337$, $p = .000$]. Taking the overall results of all the cultured genotypes receiving different hormonal treatments into account (i.e., regardless of genotype and medium composition), 1-week cold pretreatment produced the highest embryo induction rate (11.3%) from the cultured ovules, followed by freshly cultured ovules (6.49%) and 2-week cold pretreated ovules (6.43%) (Fig. 2a).

The rates of embryo induction were positively correlated with BAP concentration [$F(2,108) = 6.881$, $p = .002$]. In comparison with hormone-free medium (5.69%), 1 mg L^{-1} BAP increased the induction rate about 1.36-fold (7.78%), whereas 2 mg L^{-1} BAP almost doubled (10.75%) the gynogenesis induction (Fig. 2b).

There was a statistically significant difference between the genotypes as determined by ANOVA [$F(5,108) = 26.963$, $p = .000$]. A Tukey post hoc test revealed that among the six genotypes, the ovules of SG3 (18.15%) were the most responsive to the applied treatments, whereas the treated ovules from SG8 (0.99%), SG2 (1.24%), and SG4 (3.70%) were the least amenable (Fig. 2c). The highest rate of gynogenesis among the genotypes was recorded for SG3 (37.78%), which was induced by the interaction of 1 mg L^{-1} BAP and 1-week cold pretreatment.

Aside from the statistically significant effects of each independent variable (genotype, cold pretreatment, and hormonal treatment) on the gynogenesis, the interaction of genotype × cold pretreatment was also significant [$F(10,108) = 4.485$, $p = .000$] (Fig. 2d). In contrast, the interactions of genotype × hormonal treatment and cold pretreatment × hormonal treatment were not statistically significant [respectively, $F(10,108) = 0.592$, $p = .817$; $F(4,108) = 1.859$, $p = .123$].

The differences between the gynogenesis rate of the studied genotypes induced by the interaction of fresh/cold pretreatments with hormone-free/BAP treatments (three-way interaction) were statistically significant [$F(20,108) = 1.773$, $p = .033$]. It was mostly favored by 1-week cold pretreatment and 2 mg L^{-1} BAP. In contrast, the interaction of 2-week cold pretreatment with hormone-free medium resulted in the lowest induction rate of embryogenesis (3.53%) (Fig. 2e).

To check the gynogenesis capability loss of the ovules due to cold pretreatment, they were cultured in vitro after keeping the inflorescences for 3, 4, and 5 weeks at 4 °C. The main effect of either cold pretreatment or hormonal treatment was statistically significant [respectively, $F(5,63) = 30.982$, $p = .000$; $F(2,63) = 5.769$, $p = .005$]. However, the interaction of cold pretreatment × hormonal treatment was not significant [$F(10,63) = 1.018$, $p = .439$].

In comparison with the freshly cultured ovules, the ratio of embryo induction was doubled after 1-week cold pretreatment. The induction rate declined rapidly for the ovules pretreated for more than 1 week. Due to the very low response of the long-term cold pretreated ovules to gynogenesis, the data were only collected and presented for SG3 (Fig. 2f).

Among the structures that emerged on the gynogenesis media, 24.35% of them on the hormone-free medium and 18.98% of them on 1 mg L^{-1} BAP-containing media evolved into normal plantlets [$F(2,41) = 7.204$, $p = .002$] (Fig. 3a), and the remaining ones were vitrified [$F(2,41) = 7.102$, $p = .002$] (Fig. 3b) or became partially necrotic [$F(2,41) = 10.320$, $p = .000$] (Fig. 3c).

The follow-up medium containing 0.5 mg L^{-1} BAP resulted in some vitrification in the susceptible explants, but they managed to grow into semi-vitrified and/or normal plants (unpublished data).

The flow cytometry analysis revealed that 82.9% of the grown plantlets were haploid (Fig. 3d). To confirm the result, the chromosome numbers of a few haploid ($1n = 1x = 9$) and diploid ($2n = 2x = 18$) plants were counted under a light microscope as well (Fig. 4). Due to the labor-intensive and time-consuming workload, we did not trace the effect of the treatments on ploidy of the emerged plantlets. However, the gynogenesis rate varied among the genotypes.

Discussion

Overall ovule response is considered the most important problem in sugar beet doubled haploid production and it remains to be improved (Gürel et al. 2008). Among the many factors contributing to the rate of gynogenesis, tissue culture conditions are the ones that can be modified to obtain better results (Pedersen and Keimer 1996).

Certain stress pretreatments have a potential to redirect normal gametophytic development to the sporophytic phase. Cold, dark/light, and starvation are some of the pretreatments recommended for gynogenesis (Chen et al. 2011). The morphogenesis-stimulating effect of cold pretreatment on somatic embryogenesis (Montalbán et al. 2015), androgenesis (Eshaghi et al. 2015), and gynogenesis (Gürel et al. 2000) has been promising, although in gynogenesis it was reported to be not as

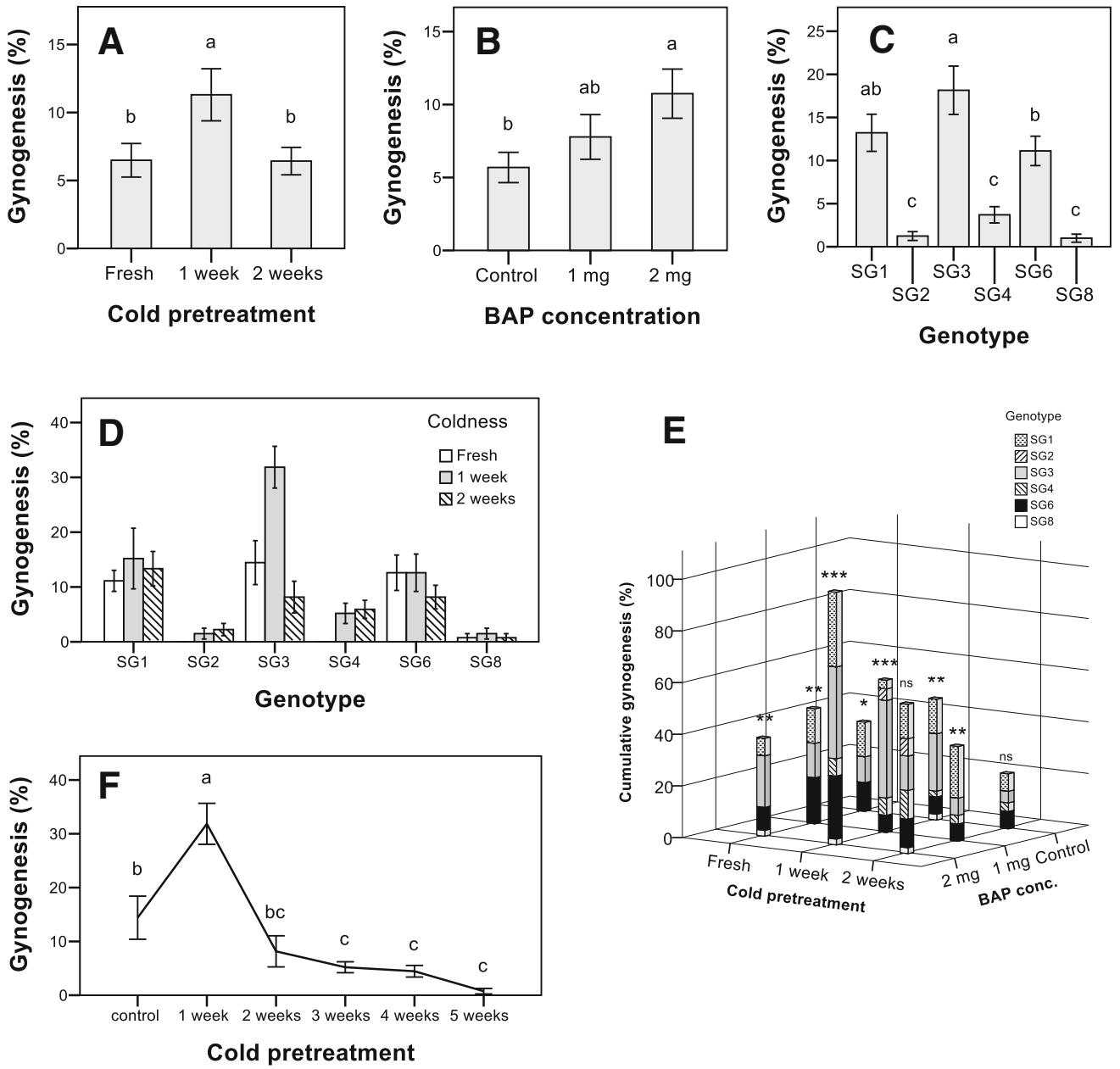

Fig. 2 Percentage of ovule gynogenesis. **a** Main effects of 1- or 2-week cold pretreatment (4 °C) of the buds or freshly cultured ovules on gynogenesis [$F(2,108) = 8.337$, $p = .000$]. **b** Main effects of hormone-free, 1 or 2 mg L^{-1} BAP on gynogenesis [$F(2,108) = 6.881$, $p = .002$]. **c** The main effect of genotype on gynogenesis ($F(5,108) = 26.963$, $p = .000$). **d** Effect of interaction of cold pretreatments and genotypes on gynogenic structures' appearance [$F(10,108) = 4.485$, $p = .000$]. **e** Effect of three-way interaction of cold pretreatments × BAP concentrations × genotypes on the gynogenesis of the cultured ovules [$F(20,108) = 1.773$, $p = .033$]. **f** Main effects of long-term (1–5 weeks) cold pretreatment on the gynogenesis [$F(5,63) = 30.982$, $p = .000$]. Means with the same letter are not significantly different from each other. *Bar* represents ± standard error (SE). ns, *, **, ***: non-significant, significance at 5, 1, and 0.1% level, respectively

effective as in androgenesis (Yang and Zhou 1982). The present report underlines the importance of cold pretreatment in combination with BAP to increase the gynogenesis rate in sugar beet.

The significant differences in the gynogenesis rate due to the main effect of genotype [$F(5,108) = 26.963$, $p = .000$] and the statistically significant interaction of genotype × hormonal treatment × cold pretreatment [$F(20,108) = 1.773$, $p = .033$] but statistically insignificant effect of hormonal treatment × cold pretreatment on gynogenesis [$F(10,63) = 1.018$, $p = .439$] indicate that genotype plays a major role in sugar beet gynogenesis. Although the method appears to be

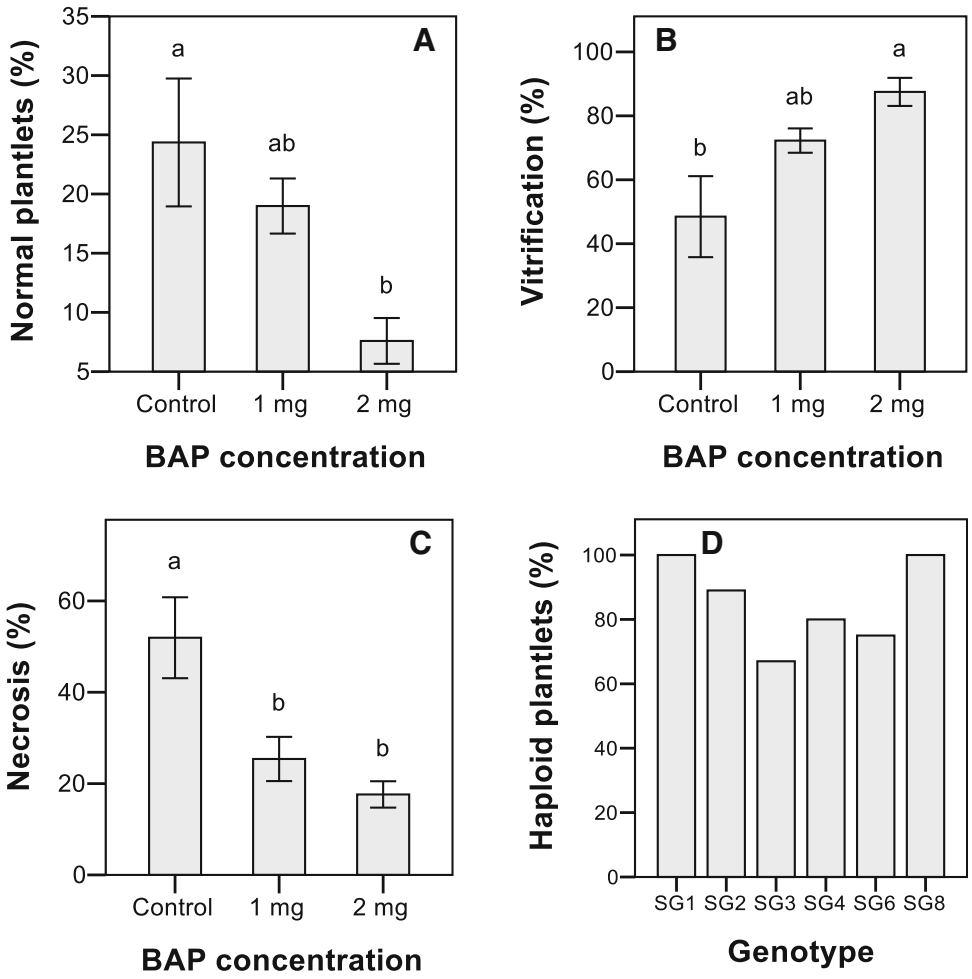

Fig. 3 Percentage of ovule gynogenesis quality and quantity. Main effects of hormone-free, 1 or 2 mg L^{-1} BAP on the percentage of gynogenic structures with: **a** normal growth [$F(2,41) = 7.204$, $p = .002$], **b** vitrification [$F(2,41) = 7.102$, $p = .002$], and **c** necrosis [$F(2,41) = 10.320$, $p = .000$]. **d** the percentage of haploid plantlets production to the in vitro cultured ovules of the genotypes. Means with the same letter are not significantly different from each other. *Bar* represents ± standard error (SE)

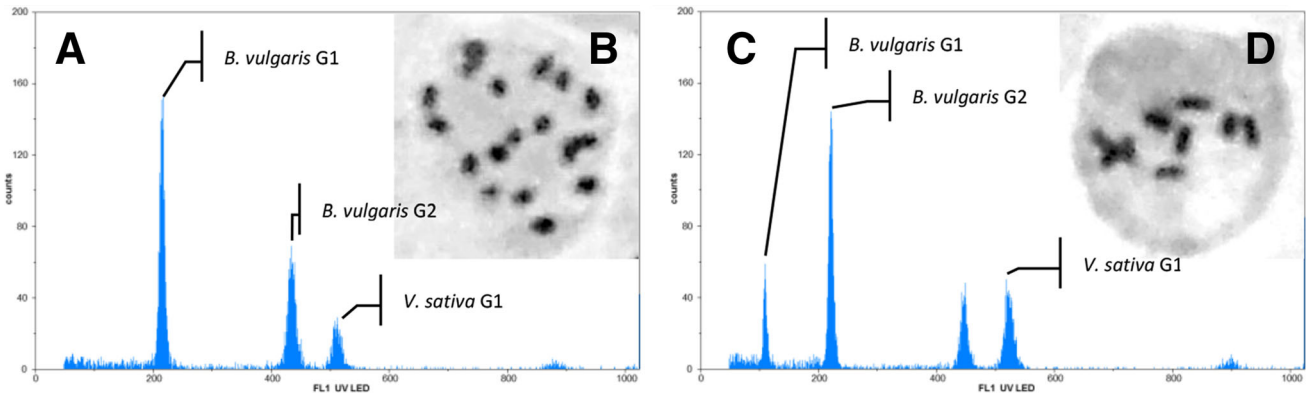

Fig. 4 Ploidy level analysis of sugar beet (*Beta vulgaris*) diploid (*A* and *B*) and haploid (*C* and *D*) plants, using flow cytometry (*A* and *C*) (common vetch *Vicia sativa* as an internal reference standard with $2C = 3.65$ pg) and light microscope (*B* and *D*). *A* A flow cytometry histogram of a diploid sugar beet. *B* Chromosomes of a diploid sugar beet using light microscopy ($2n = 2x = 18$). *C* A flow cytometry histogram of a haploid sugar beet. *D* Chromosomes of a haploid sugar beet using light micros copy ($1n = 1x = 9$)

highly genotype-dependent, its boosting effect on the average ovule response is evident. Genotype dependency, the major problem in sugar beet haploid production (Gürel 1997; Gürel et al. 2000, 2008; Gürel and Gürel 2013), has been found by other research groups for almost all genotypes attempted via in vitro methods (Doctrinal et al. 1989).

Based on our observation, cold pretreatment, particularly for 1 week, encourages embryo induction from

ovules. Regardless of hormonal treatments, keeping inflorescences at 4 °C for 1 week increased gynogenesis almost twofold compared with freshly cultured ones. As the statistical results confirm [$F(5,63) = 30.982$, $p = .000$], pretreating or storing samples for more than 1 week at 4 °C is not recommended. For example, in comparison with 1-week pretreatment, the embryogenesis rate of SG3 ovules plummeted to one-fourth, then decreased to one-sixth, one-seventh, and finally one-forty-third after being pretreated at 4 °C for 2, 3, 4, and 5 weeks, respectively (Fig. 2f).

D'Halluin and Keimer (1986) reported that pretreatment of inflorescences at 4 °C for 1 week did not change the gynogenesis rate. Weich and Levall (2003) considered keeping them for 1 week at 8 ± 2 °C quite feasible. The results from the present experiment support the stimulatory influence of cold pretreatment up to 1 week on the gynogenesis rate of sugar beet. Moreover, for the first time, a decrease in the responsiveness of sugar beet ovules to long-term cold pretreatment to induce gynogenesis was statistically indicated.

Similar to cold pretreatment in sugar beet gynogenesis, D'Halluin and Keimer (1986) did not find any positive effects of BAP at 1 or 2 mg L^{-1}; however, other researchers found that BAP treatment effectiveness depends on genotype (Lux et al. 1990; Gürel et al. 2000; Tomaszewska-Sowa 2012). The stimulatory effect of BAP in the present report is of paramount importance [$F(2,108) = 6.881$, $p = .002$], hence switching ovule development from gametophytic to sporophytic pathway was positively related to BAP concentration. The positive effect of BAP on gynogenesis was independent of genotype's main effect [$F(10,108) = 0.592$, $p = .817$]. Our observation is not consistent with Barański's (1996) report suggesting the insignificant effect of BAP at different concentrations on ovule response or Van Geyt et al.'s (1987) result that ovule induction percentage was halved by a higher ratio of cytokinins to auxins. The statistical analysis of the present experiment, for the first time suggests that BAP can have a significant effect on sugar beet in vitro gynogenesis regardless of cold pretreatment. Although the hormonal treatment (1 or 2 mg L^{-1} BAP) increased the embryo induction rate in all the cold pretreatments, their interaction was not significant [$F(10,63) = 1.018$, $p = .439$]. The main effect of BAP treatment on gynogenesis, however, was statistically significant [$F(2,63) = 5.769$, $p = .005$], which suggests that the hormonal treatment stimulatory effect on gynogenesis is independent of cold pretreatment, and hence their insignificant interaction. Despite the insignificant interaction of the cold pretreatment and the hormonal treatment on gynogenesis, their interaction with the genotypes was significant, which suggests that cold pretreatment and hormonal treatment synergy depends on genotype.

The diploid plantlets that emerged from antimitotic agent-treated ovules are considered doubled haploids without questioning the origin of ploidy levels of all the achieved plantlets (Hansen et al. 1994, 1995; 1998, 2000), but nevertheless there is always a chance of obtaining plantlets of somatic cell origin (Gürel and Gürel 1998), which may not be confidently ruled out without checking the homozygosity of the regenerants. To avoid this problem, the emerged plantlet ploidy levels should be analyzed by flow cytometry or chromosome counting before treatment for diploidization. In the present research, we achieved about 83% haploid plants, which is comparable to the result reported by Doctrinal et al. (1989) (81%). Since we used closed buds for embryo induction, achieving 100% haploid plantlets could be expected. However, 17% of the regenerants were diploid, which may be due to high levels of BAP (e.g., 1 or 2 mg L^{-1}) with a potential to regenerate plantlets from somatic tissue (Gürel and Gürel 1998), or it may induce genome duplication of the haploid regenerants (Lukaszewska et al. 2012).

Sugar beet doubled haploid production via gynogenesis is hampered by the rate of responsive ovules to embryogenesis from any desired genotype (Pedersen and Keimer 1996). The number of induced gynogenic structures in the present report is noteworthy with respect to the highest amount of emerged plantlets, which was recorded for SG3 with 37.78% emergence after 1-week pretreatment and on 1 mg L^{-1} BAP-containing medium. The results obtained from the present study showed that, depending on the genotype, the interaction of cold pretreatment and BAP effectively promoted the ovules to switch from the gametophytic to the sporophytic developmental pathway [$F(20,108) = 1.773$, $p = .033$].

We obtained one of the highest embryo induction rates from the cultured ovules in comparison with previously published results (Bossoutrot and Hosemans 1985; D'Halluin and Keimer 1986; Van Geyt et al. 1987; Doctrinal et al. 1989; Galatowitsch and Smith 1990; Lux et al. 1990; Hansen et al. 1994, 1995, 1998; Gürel et al. 2000, 2003a; Hansen et al. 2000; Weich and Levall 2003; Tomaszewska-Sowa 2012). However, in the present report, the contribution of season and genotypes to the gynogenesis rate and the in vitro induction method should not be underestimated (Doctrinal et al. 1989; Lux et al. 1990; Pedersen and Keimer 1996; Barański 1996).

Generally, by increasing cytokinins concentration in gynogenesis induction medium, an increase in the rate of haploid embryo induction can be achieved (Doctrinal et al. 1989; Lux et al. 1990). However, higher cytokinin concentrations cause vitrified to necrotic and/or malformed shoots, resulting in loss of materials or requiring considerable efforts to enable healthy plants to recover from them. While vitrification and abnormal development arise from higher doses of cytokinins, necrosis or browning/blackening stems from

phenolic compounds' oxidization of the in vitro cultured sugar beet plants (Gürel and Wren 1995b). Reportedly, activated charcoal can alleviate to some extent the problems stemming from hormonal treatment or phenolic compounds (Van Geyt et al. 1987; Gürel et al. 2000; Thomas 2008).

Explants exposed to high levels of cytokinins or treated for a long time are often recalcitrant to subsequent proliferation (Gürel et al. 2003b) and rhizogenesis (Gürel and Wren 1995a). Transferring the induced haploid embryos to a medium with a lower osmotic potential is recommended (Pedersen and Keimer 1996; Lux et al. 1990). Another choice is culturing the ovules on a lower dose of cytokinins-containing or hormone-free media. Nevertheless, it is at the expense of overall gynogenesis rate. Regrettably, losing more than half of the regenerants is generally regarded as normal in this technique, which may afflict almost all the plantlets (Pedersen and Keimer 1996; Van Geyt et al. 1987; Tomaszewska-Sowa 2012).

The negative effect of higher concentrations of BAP on the treated plants in our experiment was evident. A higher concentration of BAP may result in callus induction from ovules or dedifferentiation of some differentiated regenerants after a while (Gürel et al. 2001) and abnormal morphology of the gynogenic embryos. To cope with these types of growth, after several rounds of subculture on lower levels of the hormone, we succeeded in redifferentiating, proliferating, propagating, and letting them develop normally (unpublished data).

Therefore, depending on the genotype of plants, actually only a small fraction of induced embryos are able to develop into plants (Lux et al. 1990; Doctrinal et al. 1989; Bossoutrot and Hosemans 1985; Pedersen and Keimer 1996; Galatowitsch and Smith 1990; Tomaszewska-Sowa 2012).

Genotype dependency of sugar beet tissue culture systems is a major challenge to its in vitro breeding (Gürel and Gürel 2013). Many efforts have been made to obtain haploids from different genotypes of beet species (reviewed by Pedersen and Keimer 1996). Among them, some studies on sugar beet gynogenesis have taken advantage of highly responsive genotypes or doubled haploid plants (Pedersen and Keimer 1996; Hansen et al. 1994, 1995, 1998, 2000), which were thereafter recognized as model genotypes. The reports provided with the model or doubled haploid donor plants are considered the best; however, extrapolating from those methods to other non-model, heterogeneous, or different genotypes almost always does not produce more or less similar results.

Conclusion

Based on our observations, the haploid plantlet induction rate was improved by pretreating inflorescences for 1 week at 4 °C. When synergized with BAP supplemented into the induction medium, the result was better. However, the higher level of BAP led to higher abnormal development of emerged structures. The higher BAP concentration, the higher vitrification and the lower necrosis and normally grown plantlets. The gynogenesis induction rate is highly genotype-dependent. The main effects of cold pretreatment or hormonal treatment on inducing gynogenesis were positive, but their interaction was insignificant. The interaction of hormonal treatment with genotype was insignificant, whereas the interaction of cold pretreatment with genotype was significant. The three-way interaction of cold pretreatment × hormonal treatment × genotype was statistically significant. Cold pretreatment for more than 1 week decreased the gynogenesis rate. The results suggest that pretreatment up to 1 week and BAP treatment up to 1 mg L^{-1} can synergistically work to boost the gynogenesis induction rate.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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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

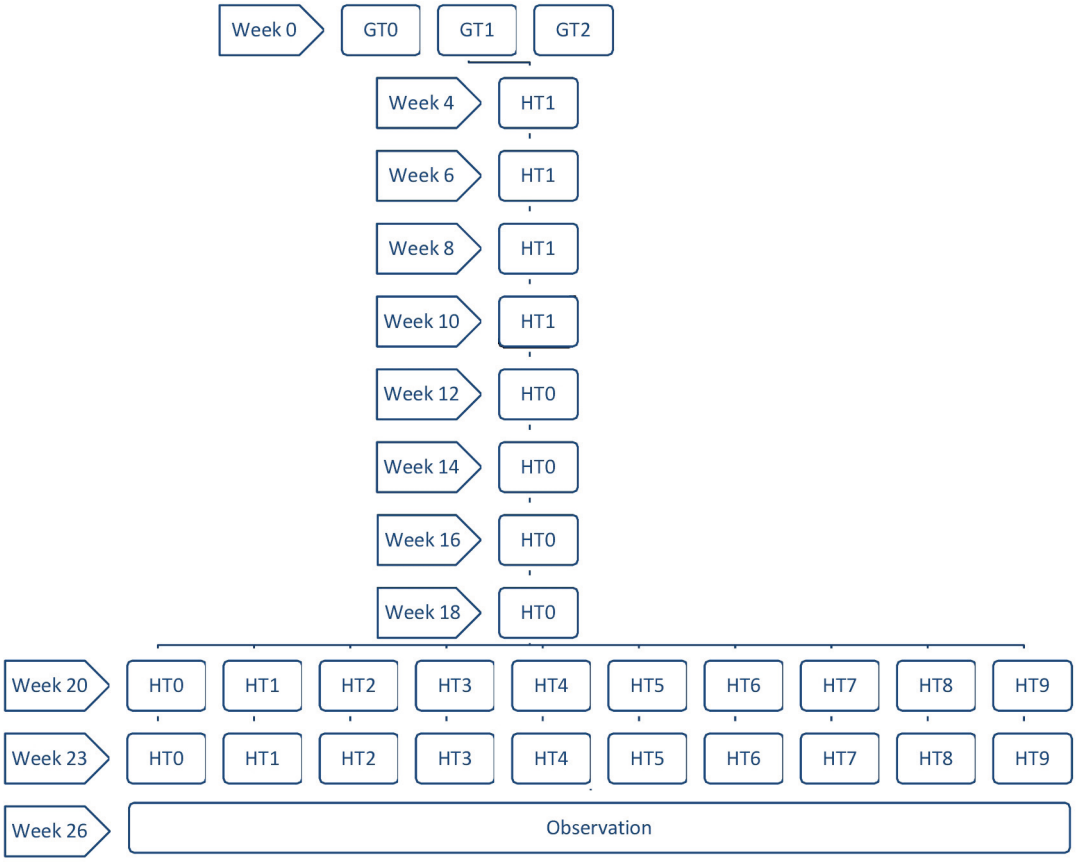

Figure 1. The sequence of hormonal treatments. The treatments are defined in Table 1. Sugar beet ovules were subjected to gynogenesis on an MS medium without hormones (GT0), or with 1 or 2 mg L^{-1} BAP (GT1 or GT2). The gynogenic plantlets were then propagated on HT1 medium (8 weeks). After that, they 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

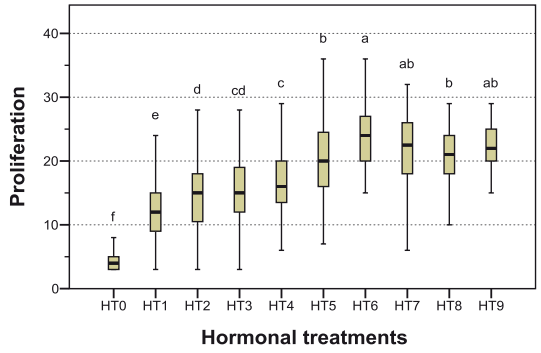

Figure 2. Effect of ten hormonal treatments on leaf proliferation (total number of leaves induced to grow from each explant) of sugar beet explants cultured *in vitro* with three leaves. The observations were made after six weeks of growing on the media defined in Table 1. The figure represents a comparison 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

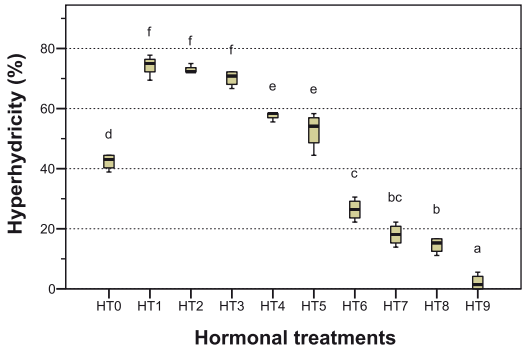

Figure 3. Effect of ten hormonal treatments on explant hyperhydricity (number of hyperhydric explants to the total number of treated explants) of sugar beet cultured *in vitro* with three leaves. The observations were made after six weeks of growing on the media defined in Table 1. The figure represents 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, necrosis. This is consistent with our findings; however we have provided an alternative medium to induce better proliferating explants.

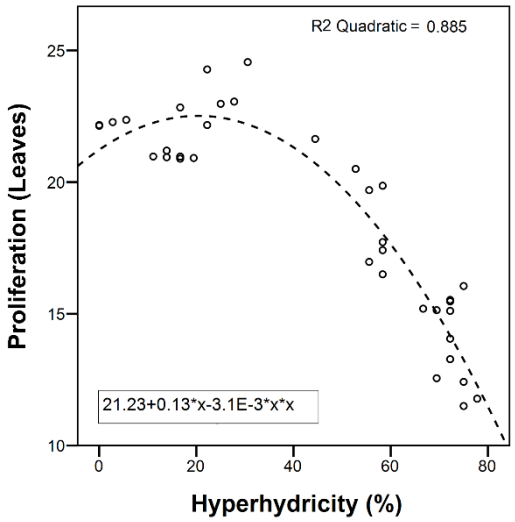

Figure 4. Correlation and regression between proliferation and hyperhydricity of haploid sugar beet explants grown *in vitro*. The two variables were negatively correlated ($\tau_b = -0.648$, n = 36, $p < 0.001$). The coefficient of determination: R^2 quadratic = 0.885, F (2, 33) = 127.23, $p < 0.001$.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

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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ORIGINAL ARTICLE

Production of doubled haploids in sugar beet (*Beta vulgaris*): an efficient method by a multivariate experiment

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Abstract The present paper describes a detailed study of a highly efficient protocol to multiply the number of haploids in sugar beet production and subsequent chromosome doubling. The protocol involves an experiment investigating factorial interactions between cold pretreatment, seven genotypes of sugar beet, and kinetin to improve haploid embryo induction. In addition, the effects of color of ovules and flower bud position on haploid embryo induction were investigated. After subjecting the data to analysis of variance or Student's *t* test ($P < .05$), the effect sizes of the independent variables were also estimated. Cold pretreatment was effective in stimulating the ovules. The haploid embryo induction rate for 1-week cold pretreated ovules (9.01%) was higher than that of freshly cultured ones (6.15%). In comparison with hormone-free medium (5.16%), the gynogenesis rate for the media supplemented with 0.05 or 0.5 mg L^{-1} kinetin increased to 7.58 and 10.05%, respectively. The genotype responses were significantly different. Interactions of kinetin × cold pretreatment, genotype × hormonal treatment, genotype × cold pretreatment, and the three-way interaction were statistically significant. Moreover, the main effects of flower bud position, ovule color, and comma-form ovule on gynogenic response were significant. After investigating the effect of 5 g L^{-1} colchicine for 3, 5, or 7 min on one genotype's (SG2) specimens, all the haploid plantlets from the other genotypes were treated for 5 min as the best treatment. The paper discusses interactions of the factors, which may be interesting for others aiming to breed doubled haploid sugar beet or possibly other related plant species.

Keywords Sugar beet · Gynogenesis · Haploid · *Beta vulgaris* · Kinetin · Ovule

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Introduction

Decades of studying doubled haploid production of sugar beet revealed it still appears recalcitrant to haploid and doubled haploid induction (reviewed in Aflaki et al. 2018). Sugar beet is normally an allogamous and self-incompatible species (Larsen 1977), which increases genetic diversity. Due to genetic variation, a universal protocol to induce a desired development from any genotype is not available. In addition, haploid induction in sugar beet cannot be done through androgenesis, the most favored method for inducing haploid embryos (Gürel et al. 2008). Therefore, researchers used an alternative method, gynogenesis (Hosemans and Bossoutrot 1983), which regenerates haploid plants through unpollinated female gametophytes. In contrast to androgenesis, gynogenesis is labor intensive and generally less efficient. However, for plants recalcitrant to androgenesis, e.g. sugar beet, or a method requiring embryo rescue, e.g. amphidiploid ovule isolation and in vitro culture, gynogenesis is worthwhile (Forster et al. 2007; Hilgert-Delgado et al. 2015).

Sugar beet gynogenesis efficiency varies between 1 and 15% and the efficiency of haploid plant growth is generally 40%, while that of doubling is considered as high as 90% (Weich and Levall 2003), but this is not supported by any other research published so far.

The two limiting factors hindering haploid production in sugar beet through gynogenesis are low response rate and genotype dependency. The former can be ascribed to the nature of the method, while the latter can stem from the very high genetic diversity of the species. To overcome the obstacles preventing haploid sugar beet production, many factors have been investigated (reviewed in Aflaki et al. 2018). Researchers suggested that genotype (Gürel 1997), growth conditions and position of inflorescences on the stalk of the donor plant (D'Halluin and Keimer 1986), developmental stage of ovules and flower bud pretreatment (Lux et al. 1990), medium composition (Hosemans and Bossoutrot 1983; Pedersen and Keimer 1996), concentration of plant growth regulators, temperature, and seasonal conditions (Doctrinal et al. 1989) could affect the response rate of sugar beet gynogenesis. In addition, some parameters were mentioned only once in the scientific literature and were not subjected to further investigation. They include color and comma form of ovules. Gürel et al. (2000) observed that $AgNO_3$ decelerated ovule browning, which they suggested might be due to inhibition of ethylene action. Van Geyt et al. (1987) reported, "Only ovules with a typical comma form reacted positively".

Pretreatment of sugar beet buds was used to increase gynogenesis rate. D'Halluin and Keimer (1986) did not observe any significant effect of cold pretreatment (4 °C for 1 week) and pre-culture (30 °C for 2 weeks) on gynogenesis rate. Lux et al. (1990) reported that cold pretreatment could induce higher number of gynogenesis event. Closed flower buds were exposed to 4 °C over 0–10 days in darkness, and the highest embryo yield was obtained over 4 or 5 days. Others indicated that cold pretreatment (4 °C) in combination with 6-benzylaminopurine (BAP) results in more induced embryos (Lux et al. 1990; Svirshchevskaya and Dolezel 2000; Gürel et al. 2000).

Sugar beet gynogenesis has been mostly increased in presence of exogenously applied phytohormones, particularly BAP (Aflaki et al. 2018). Weich and Levall (2003) recommended a combination of 0.3 mg L^{-1} BAP and 0.05 mg L^{-1} 2,4-D for an efficient induction of embryogenesis. Frequencies of embryogenesis from ovules cultured on media without hormones or supplemented with 1–2 mg L^{-1} BAP and 0.5–1 mg L^{-1} 2,4-D were 4–5%, 0, 0, and with negative effect, respectively (D'Halluin and Keimer 1986). Lux et al. (1990) used 2 mg L^{-1} (0.88 µM) BAP and 2 mg L^{-1} (9.8 µM) IBA, which induced 7.5% haploid embryo. Gürel et al. (2000) induced 7.2 and 9.6% chromosome doubling from pretreated ovules in media containing 1 or 2 mg L^{-1} BAP, respectively. Barański (1996) suggested a combination of indole-3-acetic acid (IAA, 0.5 mg L^{-1}) and BAP (0.2 mg L^{-1}) is the best for higher gynogenesis induction, whereas α-naphthaleneacetic acid (NAA) as an artificial alternative for IAA led to a lower rate of embryo induction. The numbers of haploid regenerants from treated ovules were enhanced with 0.5 mg L^{-1} IAA and 2 mg L^{-1} Kinetin or 0.2 mg L^{-1} BAP (Ferrant and Bouharmont 1994). Kinetin showed the least negative effects on endoreduplication of sugar beet. Endoreduplication can compound the results of cytogenetic analysis of gynogenic plantlet (Lukaszewska et al. 2012).

Chromosome set doubling in haploid sugar beet has not been efficient. Since the spontaneous rate of chromosome doubling in sugar beet is low (about 5%) (Weich and Levall 2003), artificial methods are used. These methods, either in vitro or in vivo, have mostly doubled less than 10% (D'Halluin and Keimer 1986; Hansen et al. 1994, 1995, 1998; Eujayl et al. 2016), occasionally more than 10% (Gürel et al. 2000; Svirshchevskaya and Dolezel 2000), and rarely beyond 50% of treated haploid explants (Hansen et al. 2000). Gürel et al. (2000) compared the effects of colchicine (50, 100, 150, 500 mg L^{-1}) and trifluralin (1.7, 3.4, 5.0 mg L^{-1}) for different durations (12, 24, 36, 48 h) and in various in vitro media on chromosome doubling of haploid sugar beet. Although statistically insignificant, they found that colchicine was more effective for doubling the chromosomes of the haploid plants than trifluralin was (25.3 and 18.2%, respectively). Hansen et al. (1998) studied the efficiency of four antimitotic herbicides (amyprophos methyl, pronamide, oryzalin, and trifluralin) on doubled haploid plant induction from cultured ovules. Amyprophos methyl at 100 µM produced the highest rate of chromosome doubling (4.7%), whereas lower doubled haploids were obtained using the other chemicals (2–3%). Bossoutrot and Hosemans (1985) used 50 and 100 mg L^{-1} colchicine in an in vitro vegetative propagation medium.

The doubling treatment was mostly applied over a few days. Doubling treatment in vitro for 48 h produced more doubled haploid explants (27.5%) than for 12 h (13.6%). However, the difference was not statistically significant (Gürel et al. 2000). Bossoutrot and Hosemans (1985) indicated that colchicine at higher concentration (100 mg L^{-1}) for 1–2 days gave the best results. The combination of the highest concentration (0.4%) for the shortest duration (5 h) was suggested as the most effective treatment with about five diploids after treating 100 ovules (Hansen et al. 1994). Hansen et al. (1995) applied the same concentration of colchicine over a shorter time (2.5 h) and found it the most efficient treatment (4.2%). However, Eujayl et al. (2016) recently applied the same method used by Hansen et al. (1995) with no success in doubling. A solution of 0.2% colchicine for 5 h (Weich and Levall 2003)

and 0.3% colchicine solution for 24 h (Svirshchevskaya and Dolezel 2000) induced 90 and 19% of doubled haploid plants, respectively. Ragot and Steen (1992) applied 0.2% colchicine to potted haploid plants by placing a cotton plug on their apical buds for 3 days and achieved a range of 30–50% of doubled haploid plants. It was also reported that applying 2–3 drops of 0.1% colchicine to the main meristem of sugar beet once a day for 3 days led to 8.4% doubled haploid plants (D'Halluin and Keimer 1986). Based on previously published research, a higher concentration of diploidizing agent over a shorter time appears to be the best treatment.

D'Halluin and Keimer (1986) observed that flowers collected from the first lateral branches showed the best gynogenesis response. Doctrinal et al. (1989) reported low yields of embryogenesis in flowers taken from the primary branches. It thus appears that the position of collected flowers may have an effect on the response rate of cultured ovules.

Plant tissue culture, in particular sugar beet haploid induction, still has room for improvement (Gürel et al. 2016; Aflaki et al. 2018). An appraisal of the scientific literature shows that doubled haploid production in sugar beet needs to be subjected to more detailed research and novel approaches, to improve the efficiency and reproducibility of reported methods as much as possible. In our previous research (Pazuki et al. 2017), the interaction of cold pretreatment and 6-benzylaminopurine (BAP) induced promising amounts of haploid structures, but the regeneration rate of haploid plantlets dropped due to the deleterious effects of the high level of BAP, which resulted in necrosis or hyperhydricity. Therefore, we decided to improve haploid induction and regeneration rate in haploid plantlets. We studied the effect of lower concentrations of kinetin in synergy with 1-week cold pretreatment in an attempt to curb the loss of haploid plantlet production, and at the same time to induce a reasonable number of haploid structures amenable to growing into haploid plantlets. During our last study (Pazuki et al. 2017), we observed an effect of flower bud on ovule response to embryogenesis treatment. However, since the position of ovules on the inflorescence was not included in the statistical design as a variable, we could not report any reliable results. Thus, in the present experiment, for the first time, flower bud position on the inflorescence was considered as an independent variable to study its effects on the gynogenesis response of the corresponding ovules. Since the eventual aim of the study was to obtain doubled haploid plants, the haploid plantlets were subjected to high concentrations of chemical treatments to double their chromosome sets.

Materials and methods

Plant material

The inflorescences (10 ± 2 cm in length) of seven diploid self-fertile sugar beet (*Beta vulgaris*) genotypes (SG1, SG2, SG3, SG4, SG5, SG7, and SG8) were collected from the last week of May to the first week of July (Etimesgut, Ankara, Turkey). The inflorescences were used either fresh or pretreated for 1 week at 4 °C in a refrigerator. After removing the bracts, 12–15 spikes were sterilized with a 70% solution of alcohol 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 four drops of Tween-20). After 30 min manually shaking, the explants were rinsed with DW three times.

Culture medium composition and condition

The common composition of the media was MS (Murashige and Skoog 1962) salts and vitamins, 30 g L^{-1} sucrose, and 3 g L^{-1} gelrite™. In addition to hormone-free medium (control), two different concentrations of kinetin (0.05 or 0.5 mg L^{-1}) were used as plant growth regulators. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The dishes containing the ovules were sealed with parafilm, and were kept in a walk-in growth chamber with an 18-h photoperiod at constant temperature of 27 ± 2 °C, irradiated under 30 ± 5 µmol m^{-2} s^{-1} of snow white extreme cool daylight fluorescent tubes (TL-D 840, Philips, Pila, Poland), at a relative humidity of 80 ± 10%.

In vitro explantation

Under a stereo microscope using forceps and scalpel, after dissecting the ovaries the ovules were removed one after another from the first closed basal flower bud toward the top flower buds, and cultured one by one in four consecutive rows on 90 × 15 mm disposable plastic petri dishes containing 25 mL of autoclaved solid induction media. The rows were denoted as top, mid-top, mid-basal, and basal segments.

The plantlets that emerged were then subcultured on a medium for proliferation and propagation containing MS salts and vitamins, 10 g L^{-1} sucrose, 6.5 g L^{-1} gelrite™, plus 0.2 mg L^{-1} kinetin to follow their growth and development into rooted plantlets in a walk-in growth chamber, irradiated with 50 ± 5 µmol m^{-2} s^{-1} using cool white fluorescent tubes (Master TL-D 840, Philips, Pila, Poland), at a relative humidity of 70 ± 10%.

Diploidization

Chromosome set doubling was done using an in vitro solidified culture medium, very similar to proliferation and propagation medium, except that 2 g L^{-1} gelrite™ was used instead of 6.5 g L^{-1} to solidify the medium. In 250 mL of DW, 5 g colchicine was dissolved, and then the solution was passed through a 22-µm filter for sterilization. The filter-sterilized solution was mixed with the cooled autoclaved medium to make a 5 g L^{-1} colchicine supplemented doubling medium. Then 45 ± 5 mL of the doubling medium was poured into each Magenta™ box. The plantlets with 3–7 leaves were subcultured on the colchicine-added medium. As preliminary tests, SG2 and SG3 plantlets were treated for 1, 2.5, or 4 h with 6, 4.5, or 3 g L^{-1} colchicine. Since the best results were observed from the explants treated with the highest concentration over the shortest time, the experiment was continued to be optimized by decreasing the treatment duration with higher concentration. However, in comparison with the other treatments showing explant necrosis, 6 g L^{-1} colchicine treatment for 1 h resulted in shoot tip necrosis. Therefore, further experiments were done with 5 g L^{-1} colchicine over different but shorter periods of treatment (45, 30, 15, or 5 min). Since the treated explants still suffered from varying degree of shoot (tips) necrosis, in the next preliminary experiment SG2 plantlets were treated for 3, 5, or 7 min. Since the preliminary results of the three treatments' duration showed that 5 min was the most effective, the other genotypes were then treated only for 5 min. After doubling treatment, the plantlets were removed from the medium and directly subcultured on the proliferation and propagation medium, where they grew and produced roots.

Flow cytometry analysis

The analysis was carried out as previously described (Pazuki et al. 2017); here it is explained briefly. Sugar beet and common vetch (*Vicia sativa*) leaf tissues were chopped all together with a sharp razor blade in a plastic petri dish 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 (Partec, Münster, Germany). To estimate the absolute value of DNA content (1C) for each sample, the analyzed samples' results were compared with an external reference (haploid/doubled haploid samples) and an internal reference (*Vicia sativa*).

Chromosome counting

The method was described previously (Pazuki et al. 2017); it is summarized here. Young leaves of in vitro plantlets or growth chamber grown plants were treated with an 8-hydroxyquinoline solution (0.002 M) for 3 h at room temperature, followed by fixation in a freshly prepared 96% ethanol:hydrochloric acid solution (2:1 v/v) for 15 min. Then they were rinsed with and kept in DW. Next, a small piece of the leaf tissue was transferred to a drop of 3% orcein in 45% acetic acid on a slide and gently pressed to squash it under a coverslip. Finally, the chromosomes were counted under a light microscope.

Acclimation

The rooted seedlings were carefully removed from the solidified media to minimize damage to the roots. The solid medium attached to the roots was cleaned manually and washed under running water. Next, they were potted in 1 L of sandy loam soil and were covered with plastic bags to prevent dehydration. The bagged explants were put in a walk-in growth chamber to acclimate them. The chamber provided an 18-h photoperiod and a constant temperature of 24 ± 2 °C, under 50 ± 5 µmol m^{-2} s^{-1} irradiation of fluorescent tubes (snow white extreme cool daylight, and TL-D 840, Philips, Pila, Poland; LUMILUX cool daylight, Osram, L 865, China, 1:1), at a relative humidity of 85 ± 10%. After 2 weeks, the plastic bags were opened gradually over the next 2–4 weeks, depending on the seedling conditions. They were watered once a week (each time 50 mL) during the first month, twice a week (each time 50 mL) during the second month, and three times a week (each time 50–100 mL) after the second month.

Observations

The rate of gynogenic embryo induction was recorded 1–2 months after ovule culture initiation. The appearance of gynogenic embryoid structures in each row on the petri dishes was documented. Apart from the quantity of gynogenesis, qualitative observations were also made to study gynogenic structure hyperhydricity, necrosis, and healthy plantlet rates affected by the three aforementioned hormonal treatments. In another preliminary experiment, the effects of ovule color (white or brown), and comma-form ovule (in a HF medium, after 1-week cold pretreatment, with six replicates) on embryogenesis were also investigated. The numbers of rooted plantlets and acclimated plants were logged. The effect of diploidization treatment on chromosome set doubling was investigated and reported. A diagram that shows different stages of the protocol is also provided (Fig. 1).

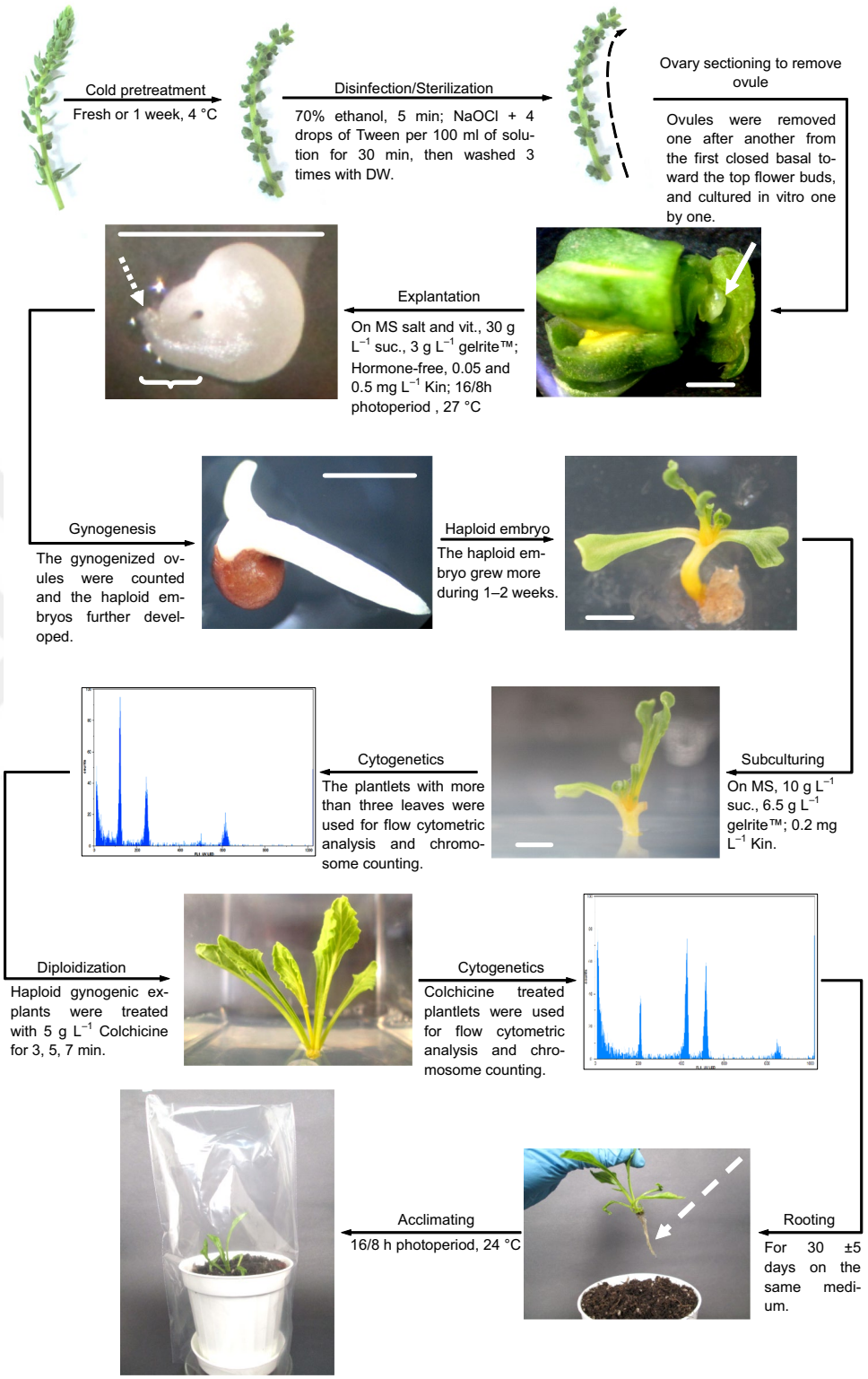

Fig. 1 Flow chart for the in vitro sugar beet doubled haploid production protocol employed during the experiment. Solid black arrows indicate transition between the steps. The dashed black arrow shows the order of ovule culture from basal flowers toward top ones. The solid white arrow indicates ovule in a dissected ovary. The dotted white arrow indicates the micropylar end. The white bracket shows ovule micropylar protrusion, comma-form. The dashed white arrow indicates rooted explant before potting. Solid white lines represent 3 mm

Experimental design

The main experiment was carried out in a completely randomized factorial design (i.e. seven genotypes × two pretreatment duration × three hormonal treatments = 42 treatments) with four (or for a few treatments three) replicates. Each replicate consisted of 16 ovules, making 64 (or 48) explants per treatment. The results were expressed as the percentage of the ovules producing embryos (the percentage of gynogenesis) and the percentage of gynogenesis events that occurred

in each of the four rows. The doubling method was applied in three replicates using 3–25 specimens in each replicate.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and means were compared using Tukey's test at 5% level of significance ($P < .05$) to check the significance between groups. The analysis results were used for estimating effect size (ES) of the independent variables by computing omega-squared (ω^2) for each of them (Cohen 1988; Field 2013). Comparisons between cold pretreatment groups, ovule color, and ovule with comma-form were done with Student's t test, the statistical analysis was two-sided, and $p < .05$ was considered significant. To estimate the ES and confidence interval (confidence level of 95%) of the independent variables, the value of a corrected measure of Cohen's d (Hedges' g) index was calculated using the means and standard deviations of the two groups (Cohen 1988; Hedges 1981). To be free from biased results, ω^2 and Hedges' g were computed for ES in the present investigation (Lakens 2013). Windows™-based SPSS® (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

Results

The present paper provides an efficient method for doubled haploid sugar beet production through in vitro ovule gynogenesis. Ovules were subjected to cold pretreatment for 1 week and then they were compared with freshly cultured ovules. The ovules were cultured on kinetin-supplemented media to increase the haploid embryo induction rate. Seven sugar beet genotypes' responses to cold pretreatment and kinetin application were investigated. The haploid explants that emerged were treated with colchicine to double their chromosome sets. The chromosome sets of the haploid and doubled haploid explants were analyzed. Finally, the doubled haploid plants were potted (Fig. 1).

The results obtained from the present experiment corroborated that cold pretreatment could efficiently induce more gynogenesis. Higher rates of haploid embryo emergence were achieved using higher concentrations of kinetin. Haploid embryo induction showed high genotype dependency. Kinetin's interaction with cold pretreatment generally induced more gynogenesis. The genotypes responded differentially to hormonal or cold treatments, hence the statistically significant interaction between genotype and hormonal or cold treatments. The three-way interaction of genotype × cold pretreatment × hormonal treatment was also significantly effective in gynogenesis induction. The flower buds taken from the basal segment produced more haploid embryos. Brown ovules showed more embryogenesis potential than white ones. Ovules removed with an intact cecum (the comma-form) had significantly higher embryo emergence. To double the chromosome set, treating the explants with 5 g L^{-1} colchicine for 5 min produced the best results (Fig. 1). The outlined results were statistically analyzed as follows.

Effect of cold treatment of inflorescences

Significantly different rates of gynogenesis were induced from fresh and 1-week cold pretreated inflorescences at 4 °C (Hedges' $g = 0.37$, 95% CI [0.05, 0.7], $t(146) = 2.29$, $p = .024$). Regardless of genotype and medium composition, 1-week cold pretreated ovules produced higher embryogenesis (9.01%) than freshly cultured ones (6.15%) (Fig. 2a).

Effect of kinetin treatment

The rate of embryogenesis increased with increases in kinetin concentration ($\omega^2 = 0.6$, $F(2, 106) = 22.05$, $p < .001$). By adding 0.05 mg L^{-1} kinetin, the gynogenesis rate reached 7.58%, whereas 0.5 mg L^{-1} kinetin almost doubled (10.05%) the induction rate in comparison with hormone-free medium (5.16%) (Fig. 2b).

Applying kinetin enabled us to improve gynogenesis quality, but it was at the expense of gynogenesis quantity. By using kinetin at lower concentrations (0.05 and 0.5 mg L^{-1}), we did not observe any hyperhydricity or necrosis.

Effect of genotypic variation

ANOVA confirmed that differences among the genotypes were statistically significant ($\omega^2 = 0.13$, $F(6, 106) = 16.78$, $p < .001$). A Tukey post-hoc test showed that among the seven genotypes the ovule response of SG1 and SG5 was statistically similar and they produced the highest rates of gynogenic embryos under applied treatments (11.46 and 11.14%, respectively), whereas the ovule response of SG7 (3.12%) was the weakest in its gynogenesis potential and produced the lowest amount of gynogenic embryos (Fig. 2c).

Effect of kinetin and cold pretreatment interaction

Interaction of 0.05 and 0.5 mg L^{-1} kinetin with 1-week cold pretreatment ($\omega^2 = 0.02$, $F(2, 106) = 7.77$, $p = .001$) induced the highest rates of gynogenic embryos among the genotypes (10.52 and 11.25%, respectively), whereas fresh/cold pretreated ovules cultured on HF and freshly cultured ovules on media containing 0.05 mg L^{-1} kinetin produced the lowest amounts of embryos (5.07, 4.39, and 5.25%, respectively) (Fig. 2d).

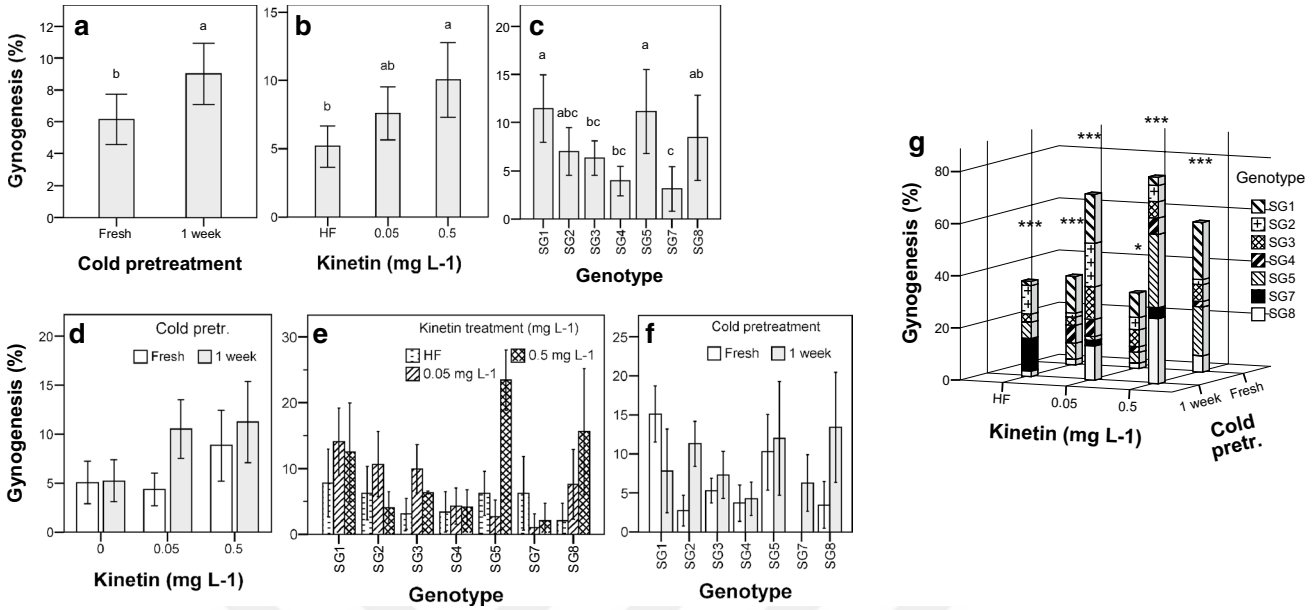

Fig. 2 Percentage of ovule gynogenesis. **a** Main effects of 1-week cold pretreatment (4 °C) or fresh culturing treatment on gynogenesis (Hedges' $g = 0.37$, 95% CI [0.05, 0.7], $t(146) = 2.29$, $p = .024$). **b** Main effects of hormone-free (HF), 0.05 or 0.5 mg L^{-1} kinetin on gynogenesis ($\omega^2 = 0.6$, $F(2, 106) = 22.05$, $p < .001$). **c** Main effect of genotype on gynogenesis ($\omega^2 = 0.13$, $F(6, 106) = 16.78$, $p < .001$). **d** Effect of cold pretreatment and hormonal treatment interaction on gynogenic embryo induction ($\omega^2 = 0.02$, $F(2, 106) = 7.77$, $p = .001$). **e** Effect of hormonal treatment and genotype interaction on gynogenesis ($\omega^2 = 0.26$, $F(12, 106) = 16.25$, $p < .001$). **f** Effect of cold pretreatment and genotype interaction on gynogenic structures appearance ($\omega^2 = 0.12$, $F(6, 106) = 15.01$, $p < .001$). **g** Effect of three-way interaction of cold pretreatment × kinetin concentration × genotype on gynogenesis ($\omega^2 = 0.14$, $F(12, 106) = 9.27$, $p < .001$). Means with the same letter are not significantly different from each other. Bar represents ± standard error (SE). Comparisons between groups were performed by using two-sided Student's t test or ANOVA (Tukey's post-analysis test). *-***: Significance at 5 and 0.1% levels, respectively

Effect of genotype and kinetin interaction

The interaction of genotype × hormonal treatment was statistically significant ($\omega^2 = 0.26$, $F(12, 106) = 16.25$, $p < .001$). The highest gynogenesis percentages among the genotypes were recorded for SG1 and SG5 (11.46 and 11.14%, respectively), for which the highest records were induced using different concentrations of kinetin. For SG1 and SG5, 0.05 mg L^{-1} kinetin and 0.5 mg L^{-1} kinetin were the best inducing concentrations (14.06 and 23.44%, respectively). Besides the two formerly mentioned genotypes, the gynogenesis percentage of SG8 was among the highest ones when it was affected by 0.5 mg L^{-1} kinetin (15.63%), which counterbalanced its very low response to HF treatment (2.08%) (Fig. 2e).

Effect of genotype and cold pretreatment interaction

The interaction of genotype × cold pretreatment was statistically significant ($\omega^2 = 0.12$, $F(6, 106) = 15.01$, $p < .001$). Except for SG1, cold pretreatment for 1 week increased the gynogenesis means of all the genotypes as compared with freshly cultured ones. This increase was more noticeable for the least responsive genotype (SG7), such that cold pretreatment induced 6.25% embryogenesis through comparison with freshly cultured ones with no response (0.0%) (Fig. 2f). However, for SG3, SG4, and SG5, the differences between gynogenesis means of fresh and cold pretreated ovules were not statistically significant. Cold pretreatment effect differed significantly between SG1, SG2, SG7, and SG8.

Effect of genotype, cold pretreatment, and kinetin interaction

The three-way interaction of genotype × cold pretreatment × hormonal treatment was statistically significant ($\omega^2 = 0.14$, $F(12, 106) = 9.27$, $p < .001$). The interaction of one-week cold pretreatment and 0.5 mg L^{-1} kinetin was the best combination and induced the highest rate of gynogenic embryos from the genotypes, but this rate varied between different genotypes. The interaction of HF treatment with both cold pretreatments and the interaction of freshly cultured explants with 0.05 mg L^{-1} kinetin were the least favorable combinations for the genotypes as a whole (Fig. 2g). The best records of haploid embryogenesis were seen for SG5, SG8, and SG1 (28.1%, 25%, 21.9%; 95% CI [24.6, 31.6; 20.9, 29.1; 18.4, 25.4] respectively), whereas freshly cultured SG7 at both hormonal treatments and cold pretreated SG4 in HF medium had the worst records (0.0% for all of them).

Effect of flower bud position

The effect of flower bud position on gynogenic response was significant ($\omega^2 = 0.064$, $F(3, 424) = 19.2$, $p < .001$). It was examined by removing the buds from the first closed basal flower bud toward the top one and explanting in four rows on plates to keep records of each segment of the flowering stem. However, the effect of row interaction with other factors in all types of combinations was not significant (data not shown). The buds taken from the basal segment were the largest contributors to haploid embryo induction, because they showed higher rates of redirecting normal gametophytic development to the sporophytic phase. However, the buds excised from the top and mid-top segments made the least contributions to the overall gynogenesis (Fig. 3a).

Effect of ovule color

Ovule color effect (white or brown) on embryogenesis potential was statistically significant (Hedges' $g = 0.671$, 95% CI [0.17, 1.17], $t(146) = 2.68$, $p = .008$) (Fig. 3b). The ovules removed and explanted from SG7 (with the least amount of gynogenesis) stayed whitish even after two months (Fig. 3c), in spite of the fact that the color of the other genotypes' ovules turned from white to brown/dark brown after 1–2 weeks (Fig. 3d).

Effect of comma-form ovule

Our investigation into the embryogenesis of the ovules taken from SG2 during a preliminary experiment showed that the ovules should be carefully removed from the ovaries to keep their comma-form intact. Otherwise, the rate of embryogenesis can be markedly decreased. The effect of comma-form on haploid embryogenesis was very significant (Hedges' $g = 2.548$, 95% CI [1.025, 4.071], $t(10) = 4.781$, $p = .001$). The mean rate of gynogenesis for the ovules with an invisible or broken comma-form was 1.04%, but for the ovules with comma-form it was 6.25%. Therefore, to maintain the gynogenesis potential of the cultured ovules, except for the preliminary experiment, for the main experiment all the ovules were cultured with comma-form (Fig. 1).

Growth of explants into potted plants

The gynogenic normally-grown embryos were then subcultured on a medium containing MS salts and vitamins, 10 g L^{-1} sucrose, 6.5 g L^{-1} gelrite™, plus 0.2 mg L^{-1} kinetin. Low concentrations of kinetin and sucrose and high concentration of gelrite™ helped the subcultured plantlets to grow without any signs of hyperhydricity. The same medium was also used for colchicine-treated explants.

Moreover, all the plantlets were rooted easily (data not shown). The rooted seedlings were potted and were covered with plastic bags to acclimate them. The survival rate of the plants was 98%.

Effect of haploid chromosome set doubling treatment

Doubling treatment using a solidified medium containing 5 g L^{-1} colchicine for 3, 5, and 7 min was firstly examined on a group of plantlets propagated from the SG2 genotype (Fig. 4). This preliminary examination showed that differences among the three durations of doubling treatment were statistically significant ($\omega^2 = 0.67$, $F(2, 6) = 10.33$, $p = .011$). Treating for 5 min was the best, while the two other treatments (3 or 7 min) were not very effective (Fig. 4e). By considering the result of the preliminary examination, for all the genotypes only 5-min colchicine treatment was employed. SG3 and SG2 had the highest diploidization rate, whereas

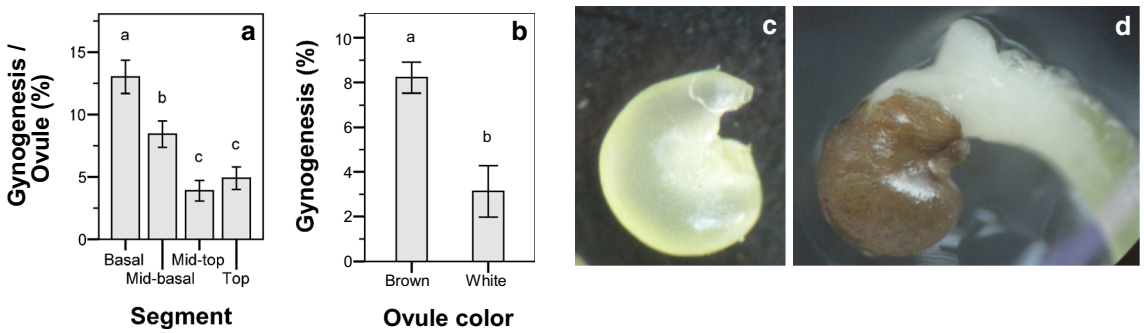

Fig. 3 Effects of ovule color and ovule position on inflorescence on gynogenesis. **a** The ovules removed from basal segment of inflorescences produced more gynogenic embryos than upper segments ($\omega^2 = 0.064$, $F(3, 424) = 19.2$, $p < .001$). **b** Brown ovules produced more gynogenic embryos than white ones (Hedges' $g = 0.671$, 95% CI [0.17, 1.17], $t(146) = 2.68$, $p = .008$). Means with the same letter are not significantly different from each other. Bar represents ± standard error (SE). Comparison between groups was performed with ANOVA at 5% level of significance (Tukey's post-analysis test, $P < .05$). **c** Represents a white ovule. **d** Represents a brown ovule (the white emergent is a gynogenic embryo emerging from the micropylar end of the ovule)

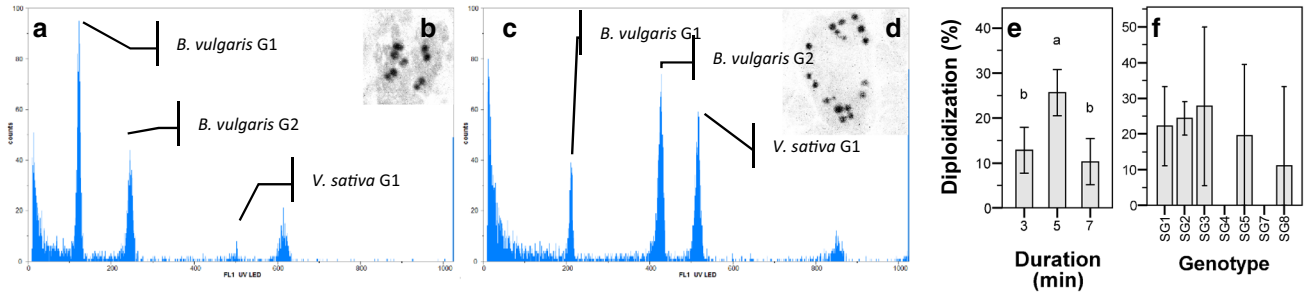

Fig. 4 Ploidy level analysis of sugar beet (*Beta vulgaris*): haploid (**a, b**) and doubled haploid (**c, d**) plants, using flow cytometry (**a, c**) (common vetch *Vicia sativa* as an internal reference standard with 2C = 3.65 pg) and light microscope (**b, d**). **a** A flow cytometry histogram of a haploid sugar beet. **b** Chromosomes of a haploid sugar beet pictured using a light microscope (1n = 1x = 9). **c** A flow cytometry histogram of a doubled haploid sugar beet. **d** Chromosomes of a doubled haploid sugar beet pictured using a light microscope (2n = 2x = 18). **e** The result of a preliminary test for doubling efficiency done on the plantlets propagated from the SG2 genotype. The explants were treated on a solidified medium containing 5 g L^{-1} colchicine for 3, 5, and 7 min. The differences among the three doubling treatment durations were statistically significant ($\omega^2 = 0.67$, $F(2, 6) = 10.33$, $p = .011$). Treatment for 5 min was the most preferred. **f** Following the preliminary test, all the genotypes were treated only for 5 min with 5 g L^{-1} colchicine. However, the diploidization results were not significantly different ($F(6, 14) = 2.95$, $p = .084$)

SG4 and SG7 chromosome sets were not doubled (Fig. 4f). However, the diploidization rates recorded for the genotypes were not significantly different ($F(6, 14) = 2.95$, $p = .084$).

Discussion

The effect of cold pretreatment and its interaction with kinetin were investigated in the present experiment. After decades of attempts at sugar beet haploid induction (Levan 1945), sugar beet gynogenesis rate is still very low (Aflaki et al. 2018). Researchers suggest that tissue culture conditions can be modified to obtain better results in haploid embryogenesis (Pedersen and Keimer 1996).

Normal gametophytic development of plants can be stimulated to develop into the sporophytic phase. Some abiotic stress pretreatments, e.g., cold, dark/light, and starvation, have shown stimulating effects (Chen et al. 2011). Cold pretreatment's effect on seed germination (Landi et al. 2016), gametic and somatic embryogenesis (Cardoso et al. 2016), androgenesis (Popova et al. 2016), and callogenesis (Rout et al. 2016) was reported. Yang and Zhou (1982) suggested that cold pretreatment is not effective for gynogenesis. Cold pretreatment's effect on in vitro gynogenesis of sugar beet was reported to be statistically insignificant by D'Halluin and Keimer (1986). Others found that the same pretreatment was statistically significant for inducing haploid embryogenesis (Lux et al. 1990; Svirshchevskaya and Dolezel 2000; Gürel et al. 2000); however, it has not been fully investigated.

We have previously studied the effect of cold pretreatment over 1–5 weeks (Pazuki et al. 2017), which showed that cold pretreatment for 1 week is the best treatment. Therefore, for the present experiment, the ovules were cold treated for 1 week to compare them with freshly cultured ones (untreated). Student's *t* test confirmed that there was a statistically significant difference between the pretreatment effects on haploid embryogenesis means ($t(146) = 2.289$, $p = .024$). The ES of cold pretreatments was computed by adjusting the calculation of pooled standard deviation with weights for the sample sizes. An ES of Hedges' $g = 0.37$, 95% CI [0.05, 0.7] for the treatments suggests that cold pretreatment for 1 week has a small effect on gynogenesis (Cohen 1988; Hedges 1981).

The main effect of hormonal treatment was statistically significant and increased the embryo induction rate in interaction with cold pretreatment ($F(2, 106) = 22.05$, $p < .001$). In natural conditions, the ovule is nourished by the sap provided by the ovary. Apart from macro- and micro-elements, ovule growth and development are dependent on phytohormones. They are programmed to evolve into haploid gametophytes, and diploid sporophytes after fertilization. In contrast, by gynogenesis, the aim is redirecting an ovule's natural development toward haploid development with sporophytic growth. While providing ovules with macro- and micro-elements is essential for their survival, stimulating phytohormone supplementation is suggested to increase the very low response of haploid embryo induction in this species. Very low quality and quantity gynogenic embryos in sugar beet are considered normal (Pedersen and Keimer 1996). The percentage of viable plantlets with the potential to grow normally can be very low (Eujayl et al. 2016). However, the hormonal treatments we used showed beneficial effects. Kinetin at 0.5 mg L^{-1} almost doubled the induction rate (10.05%), markedly more than the result from another report, which was as low as 0.5% (Eujayl et al. 2016). Moreover, we did not observe any hyperhydricity or necrosis. In addition to

the p value, the ES for the hormonal treatments was also calculated (Cohen 1988). An ES of $\omega^2 = 0.6$ suggested that kinetin had a medium effect on haploid embryogenesis of the ovules. Therefore, according to the statistical analysis, the hormonal treatments effectively and significantly improved both the quantity and quality of haploid embryo induction.

The main effect of genotype on gynogenesis was statistically determining. In addition, the response of the genotypes to the treatments was significantly different, which suggests that the protocol described here is genotype dependent. Genotype dependency is the major problem in sugar beet gynogenesis (reviewed in Gürel et al. 2008; Gürel and Gürel 2013; Aflaki et al. 2018). This problem may arise from the allogamous nature of the plant, which results in heterogeneity. The amount of variation in *Beta* species with a great tendency for crossbreeding may be the reason behind the genotype dependency of protocols. However, the results confirmed that although the method is genotype-dependent its positive effects on gynogenesis are evident. ANOVA suggested that genotype had a statistically significant effect on gynogenesis ($\omega^2 = 0.13$, a medium ES) and did not happen by chance ($F(6, 106) = 16.78$, $p < .001$). By overcoming the genotype dependency of the protocol, its efficiency can be increased. Seemingly, genotypes in *Beta* species are very recalcitrant to respond to a defined method; therefore, modifying the available methods to boost their efficiency as much as possible may be more feasible.

The cold pretreatment and hormonal treatment interaction statistically had a positive effect on gynogenic embryo induction ($F(2, 106) = 7.77$, $p = .001$). Therefore, this two-way interaction could actually increase haploid embryo induction. Cold pretreatment did not show any effects without hormonal treatment (HF). In addition, after 1-week pretreatment, the ovules cultured on HF medium responded less than the fresh ones did. This can be ascribed to the depletion of nutrients and endogenous phytohormones, which could be provided by exogenous alternatives. The media supplemented with kinetin showed very promising results in terms of interaction with the cold pretreatment. The ES of this interaction was small ($\omega^2 = 0.02$). However, 0.05 mg L^{-1} kinetin was not capable of improving the response of the freshly explanted ovules, which could be compensated for by 1-week cold pretreatment. The higher concentration of kinetin (0.5 mg L^{-1}) boosted the efficiency of both cold pretreatment groups. Lux et al. (1990) reported the effect of cold and BAP interaction partially, and we have investigated the same treatments' effects in detail (Pazuki et al. 2017). In both experiments, the detrimental effects of BAP on gynogenic embryo quality and viable plantlets were evident. In the present experiment, in addition to corroborating the beneficial effects of cold pretreatment, we showed that cold pretreatment in interaction with kinetin instead of BAP could improve gynogenic embryogenesis in quality, although it is partially at the expense of quantity.

Genotype alone had a medium effect on haploid embryo production ($\omega^2 = 0.13$). Its ES was sufficient to influence both types of two-way interaction (Fig. 4e, f), which means that the response of the genotypes to the hormonal treatment varied significantly ($F(12, 106) = 16.25$, $p < .001$). This interaction, however, had a larger ES than genotype alone ($\omega^2 = 0.26$ vs. $\omega^2 = 0.13$, respectively). In comparison with previously published papers that investigated the interaction of genotypes with BAP (Lux et al. 1990; Gürel et al. 2000), the present paper reports in detail the ES of kinetin's interaction with genotypes. In agreement with the published papers, our results confirmed that the response of genotypes to hormonal treatment and cold pretreatment could differ.

The genotype responses in a two-way interaction with the cold pretreatment were always favorable, except for SG1, which showed the opposite effect. The different response of SG1 to the interaction can be attributed to the differential effects of season on genotypes (Barański 1996; Pedersen and Keimer 1996). However, 1-week cold pretreatment effect in interaction with genotype was generally better than the fresh treatment. The results are consistent with those in previously published papers (Lux et al. 1990; Gürel et al. 2000). Additionally, the ES of the interaction was medium ($\omega^2 = 0.12$), which means that this interaction can be confidently supposed ($F(6, 106) = 15.01$, $p < .001$).

The three-way interaction of the independent variables (genotype × cold pretreatment × hormonal treatment) showed statistically significant effects ($F(12, 106) = 9.27$, $p < .001$), and its ES on gynogenesis was medium ($\omega^2 = 0.14$). This shows that the interaction was very effective in inducing haploid embryogenesis, although responses of the genotypes to the interaction were dissimilar. This interaction has not been investigated in depth before the present experiment (Lux et al. 1990; Gürel et al. 2000). However, our results strongly suggest that this combination can be considered for sugar beet haploid induction through ovule explanting.

Our recent investigation into the effect of BAP on sugar beet gynogenesis showed that although the gynogenesis rate may be higher than that of kinetin most of the haploid emerged structures could be hyperhydric or even necrotic (Pazuki et al. 2017). Therefore, we decided to carry out a new experiment using kinetin to examine the possibility of curbing hyperhydricity and necrosis. The plantlets that emerged in kinetin-containing media were all free of any symptoms we had seen in the presence of BAP. Often, in the published papers on sugar beet gynogenesis, the effect of the treatment applied on the quantity of haploid structures emergence is highlighted, but the quality of the emerged plantlets is not underlined. This is in spite of the fact that among many gynogenic structures only a mere fraction of induced embryos are able to develop into plantlets (Lux et al.

1990; Doctrinal et al. 1989; Hosemans and Bossoutrot 1983; Bossoutrot and Hosemans 1985; Pedersen and Keimer 1996; Galatowitsch and Smith 1990; Tomaszewska-Sowa 2012; Eujayl et al. 2016), which suggests emergence of defective embryos.

D'Halluin and Keimer (1986) suggested that the flowers collected from the first lateral branches had the highest potential for gynogenesis, and Doctrinal et al. (1989) observed that the flowers taken from the primary branches had the lowest. During our last experiment (Pazuki et al. 2017), we noted that even the closed buds excised from the same branch had different capabilities to undergo gynogenesis. At that time, we could not test our hypothesis because the ovules had not been cultured appropriately in a statistical design. However, in the present experiment, we excised and cultured the ovules in vitro to trace their positions and to record their responses. We ascribe the flower position effect ($\omega^2 = 0.064$, medium ES) to the endogenous auxin and cytokinin effects on the ovules. The published literature suggests and our results confirm that cytokinins, e.g., BAP or kinetin, induce gynogenesis and auxins reduce gynogenesis (Lux et al. 1990; Barański 1996). Auxins have a downward flow from apex to root, and cytokinins flow generally from root to shoot. Therefore, the higher potential of the basal ovules for gynogenesis and the lower potential of the ovules from the top segments can be attributed to the higher amounts of auxin in the top segments and the higher amounts of cytokinins in the basal segments. The results indicate that higher exogenously applied kinetin could counteract the inhibitory effect of endogenous auxin.

It was found that ovule browning speed could be decreased by applying $AgNO_3$ (Gürel et al. 2000). We observed that all the ovules from all the genotypes (except for SG7) turned brown after 1–2 weeks. However, the ovules from SG7 remained white even after 2 months. This genotype produced the lowest amount of gynogenic embryos. Ovule color after explanting had a medium ES on gynogenesis rate (Hedges' $g = 0.671$, 95% CI [0.17, 1.17], $t(146) = 2.68$, $p = .008$).

A publication on sugar beet ovule gynogenesis merely mentioned that comma-form ovules were responsive (Van Geyt et al. 1987). We tested it in a statistically deigned experiment. Our results showed that the ES of comma-form was very large (Hedges' $g = 2.548$). Bossoutrot and Hosemans (1985) observed that in an ovule the root pole was oriented toward the micropylar end and the apical pole toward the body of the ovule. Since a truncated ovule loses its cecum (Ferrant and Bouharmont 1994), which contains the egg cell, depending on the amount of truncation, gynogenesis will be proportionately decreased.

Colchicine at higher concentrations seems more effective. Hansen et al. (2000) and Gürel et al. (2000) reported that the highest concentration of polyploidizing agent, 0.12 g L^{-1} amiprophos-methyl for 2 h and 0.5 g L^{-1} colchicine for 2 days, respectively, produced more doubled haploid plants. It is notable that we examined a very high concentration of colchicine over a very short time. The short treatment with very high colchicine concentration was in fact effective ($\omega^2 = 0.67$, a medium ES). Since the doubling protocol described here can be done in a very short time, it can be of great interest for others to consider. The rates of diploidization for the genotypes were not significantly different ($F(6, 14) = 2.95$, $p = .084$), which can be ascribed to the large differences of intra-genotypic response to the doubling treatment (see ± SD bars in Fig. 4f).

Conclusion

The present paper reports a detailed multivariate experiment on sugar beet haploid and doubled haploid induction/production. Based on our results, cold pretreatment for 1 week, kinetin for 0.05 or 0.5 mg L^{-1}, in interaction with genotypes can be an effective strategy to consider for improving the efficiency of gynogenesis. Apart from the two–three-way interactions (cold pretreatment × hormonal treatment × genotype), all the treatments showed their inducing effect as the main variables. We examined the effect of ovule color on gynogenesis, which was statistically significant. In addition, we showed that the position of the flower bud on the inflorescence could have an effect on gynogenesis. By taking all of these independent variables into account, one may be able to increase haploid embryo yield up to fivefold. The doubling treatment protocol we used was one of the simplest and the most efficient methods, done in a short time (5 min) with an efficiency of 25% doubled haploid induction. The acclimation rate for the surviving and doubled plants was 98%. In the present study, we investigated many factors' effects on haploid embryo induction and doubled haploid plant production from sugar beet. The results presented are statistically analyzed and confirmed. In addition, the discussion can be of help to others trying to produce doubled haploid plants from sugar beet or likely other plants through gynogenesis.

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Author contributions SG, EG, and AE designed and outlined the research. AP and FA performed the experiment, analyzed, interpreted data, and wrote the manuscript. SG, EG, and AE edited it.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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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 ± 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

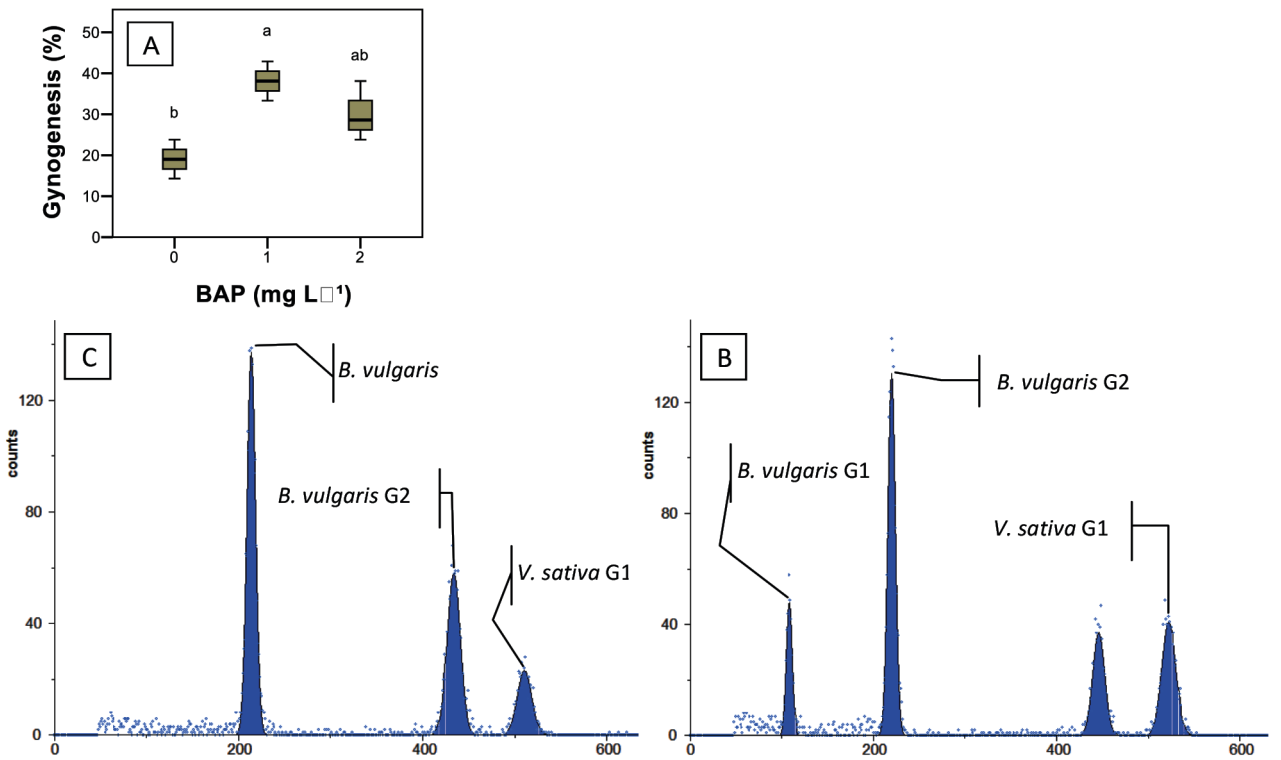

Figure 1. Gynogenesis and ploidy level analysis of sugar beet (*B. vulgaris*). A) Three hormonal treatments, i.e. hormone-free (HF) or 1 or 2 mg L^{-1} BAP, were applied to induce gynogenic embryos from a sugar beet genotype. B) A flow cytometry histogram of haploid and C) doubled haploid sugar beet. 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 $\varepsilon^2 = 1.0$. 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

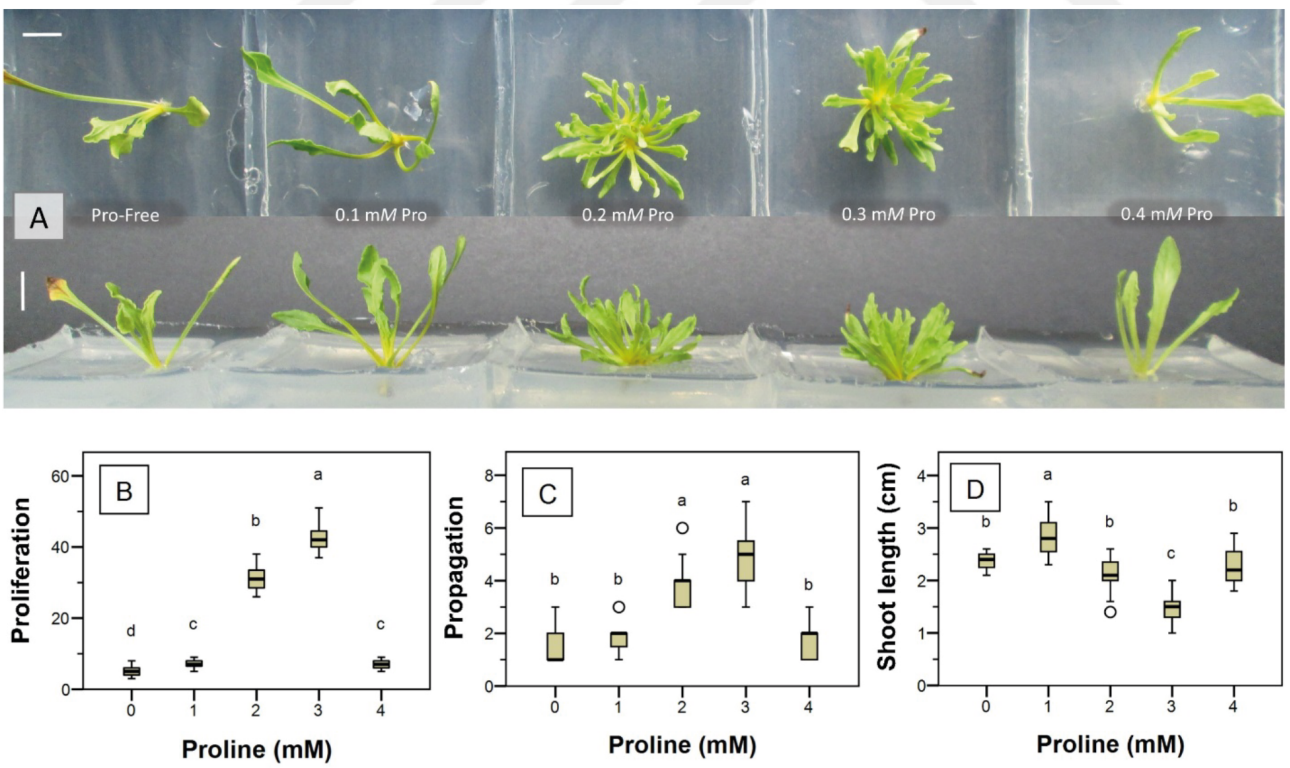

Figure 2. Effects of different proline concentrations on proliferation, propagation, and shoot length. A) Effects of 0, 0.1, 0.2, 0.3, and 0.4 mM proline on the dependent variables are shown (bar = 1 cm). B) Treatment effects on proliferation, C) propagation, D) and shoot elongation. The heavy black 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.

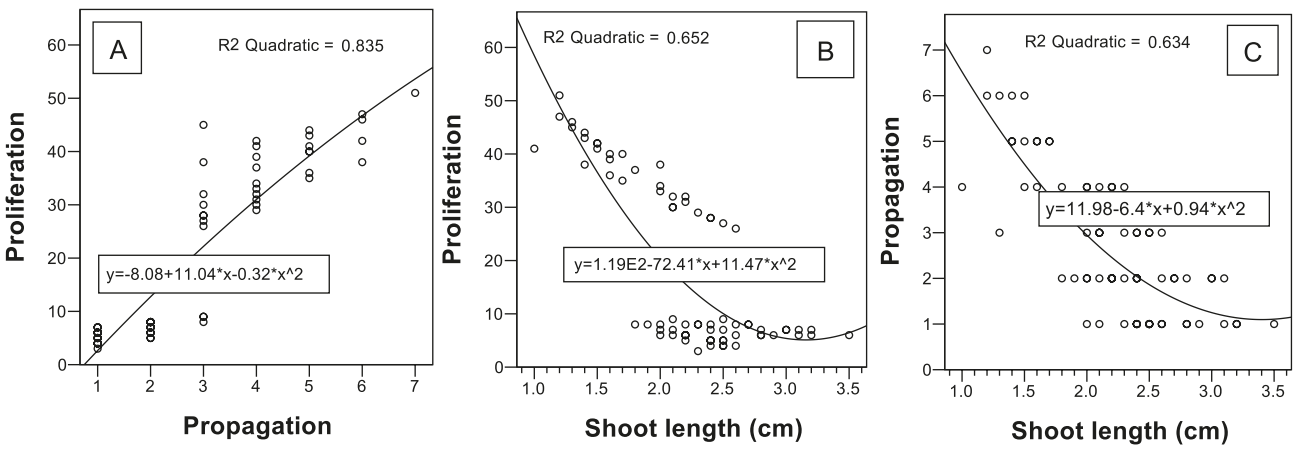

Figure 3. Bivariate correlation coefficient between proliferation, propagation, and shoot length. A Kendall's tau-b correlation was run to determine the relationships between the dependent variables: A) proliferation and propagation, B) proliferation and shoot length, and C) shoot propagation and length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

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