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Stomatal Morphology and Desiccation Response of Persian Walnut Tissue Culture Plantlets Influenced by the Gelling Agent of *In Vitro* Culture Medium

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A R T I C L E I N F O

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A B S T R A C T

There are diverse types of gelling agents that are used in media cultures. Agar and Gelrite are among the gelling agents used in DKW culture medium, as the common culture medium for the micropropagation of walnut plants. The effects of these gelling agents have not been investigated on the successful production of *in vitro* explants and the response of the explants to *ex vitro* evaporative demand is overlooked so far. Stomata and water relations of tissue culture medium determine the successful production of *in vitro* plants, therefore this experiment was conducted to investigate the effect of two types of gelling agents (Agar and Gelrite) on the stomatal characteristics, transpiration rate (E), and desiccation responses of *in vitro* walnut explants. Stomatal morphology, transpiration rate, RWC, and some morpho-physiological traits such as shoot length, chlorophyll content, osmotic potential (ψs), proline, and glycine betaine content were evaluated in micropropagated walnut explants cultured on Agar or Gelrite. Analysis of results indicated no considerable changes in the morpho-physiological characteristics of explants grown in DKW medium containing Agar or Gelrite gelling agents. Compared with the medium containing Agar, adding Gelrite to the DKW medium caused a decrease in E and an increase in relative leaf water content (RWC) of the walnut explant's leaves during desiccation. Gelrite induced generation of more closed stomata leading to a reduction in E and increase in RWC during desiccation. This resulted in improvement of walnut plantlet's capacity to conserve their water content and as the consequence promoted ability to prevent *ex vitro* wilting.

Introduction

Propagation by seeds causes high genetic diversity and segregation in walnut plants. *In vitro* propagation of walnuts has emerged as a rapid approach for mass propagation of cultivars with desirable traits and for production of high quality, disease-free, and uniform plant materials (Vahdati *et al.*, 2004; Gandev, 2014). Among different culture media for micropropagation of walnut including MS (Murashige and Skoog Medium), WPM (Woody Plant Medium) and DKW (Driver-Kuniyuki Walnut Medium) (Driver and Kuniyuki, 1984; Saadat and Hennerty, 2002; Payghamzadeh and Kazemitabar, 2011), the DKW culture medium has been developed specifically for walnuts (Driver and Kuniyuki, 1984).

There are diverse types of gelling agents that are used in culture media for micropropagation. The most popular ones are Agar and Gellan gums, natural polysaccharides that can gel at room temperature (Masondo *et al.*, 2015). The choice of gelling agent is very important for the regeneration of tissue culture

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plants. Agar is the most popular gelling agent and the reason for its widespread use is its stability, gel transparency, and high ability to prevent the digestion of plant enzymes (Masondo *et al.*, 2015; Seifeldin and Ozzambak, 2016). Despite of several benefits of Agar, increasing the concentration of this substance may adversely affect the growth of *in vitro* plants. At high concentrations it hardens the medium and does not allow efficient uptake of nutrients by the plant tissues (Seifeldin and Ozzambak, 2016). Agars are derived from seaweed (Agarophytes) and contain polysaccharides related to galactans. On the other hand, Gellan gum is obtained from bacterial (*Pseudomonas elodea*) polysaccharides consisting glucuronic acid, rhamnose, and glucose. There are quantitative and qualitative differences for inorganic fractions and physicochemical gel characteristic between Agar and Gellan gum (Klimaszewska *et al.*, 2000). Despite of all these differences, Gelrite (often 0.2%) is one of the best alternatives to Agar, because Gellan gum is easily gelled in the presence of monovalent or divalent cations and is more economically appropriate than Agar, since approximately half of them are required to attain the equivalent gelling strength as Agar. They also produce very clear gels in the absence of contaminants (Masondo *et al.*, 2015; Seifeldin and Ozzambak, 2016). However, the use of gellans, including Gelrite, often causes hyperhydricity or vitrification (Van den Dries *et al.*, 2013; Masondo *et al.*, 2015).

Hyperhydricity is a physiological disorder that results in generation of translucent, glassy, and succulent leaves and stems, curled and wrinkled leaves, which can affect the regenerative ability and transfer phase of *in vitro* plants (Ziv, 1991; Franck *et al.*, 2004; Van den Dries *et al.*, 2013). It seems gelling agent such as Gelrite increases the absorption of hyperhydricity stimulants (cytokinins, ammonium ions, and water) (Franck *et al.*, 2004). There are several factors that can cause hyperhydrosis, including high relative humidity, gas accumulation and high ethylene concentration in the atmosphere of culture vessels, decreased protein and chlorophyll content, light filtration through the flask, high cytokinin application, and poor cell wall development (Ziv, 1991; Franck *et al.*, 2004; Hassankhah *et al.*, 2014). There are so many reports suggested that hyperhydricity is also the result of a low concentration of gelling agent (Ziv, 1991; Seifeldin and Ozzambak, 2016) and liquid or low concentration of Agar in culture medium, which induce callus formation and disrupts cellulose biosynthesis (Ziv, 1991).

Challenges with seed propagation such as long juvenility, high degree of heterozygosity, and sometimes self-incompatibility (Grouh *et al.*, 2011; Farsi *et al.*, 2018; Thapa *et al.*, 2021), encourage horticulturists to use *in vitro* propagation as a rapid and large scale method of walnut propagation, however, high mortality of *in vitro*-generated plantlets during transfer to *ex vitro* condition is a major limitation for plant micropropagation (Pospisilova *et al.*, 2007; Eshghi Khas *et al.*, 2020). Proper stomatal functioning is considered as one of the main factors influencing the acclimatization and successful transfer of *in vitro* plants. Disturbance in normal stomatal morphology and its functioning and poor cuticular development are the main cause of excessive water loss of *in vitro*-generated plants (Santamaria *et al.*, 1993; Hazarika, 2006). *In vitro* environment is characterized with high relative humidity. Plants developed in high relative humidity have abnormal morphology, anatomy, and physiology, which are often characterized with poor photosynthetic efficiency, malfunctional stomata, and reduced cuticle development on their leaves (Rezaei Nejad and van Meeteren, 2005; Aliniaeifard and van Meeteren, 2013; Asayesh *et al.*, 2017a; Asayesh *et al.*, 2017b). Plants produced in *in vitro* environment that are different from the normal growing conditions of plants in greenhouses or field, often failed to control their transpiration and water loss, leading to reduced leaf capacity to conserve water and consequently wilting and desiccation (Aliniaeifard *et al.* 2020; Fanourakis *et al.*, 2013;

Aliniaeifard *et al.*, 2014; Aliniaeifard and van Meeteren, 2014; Vahdati *et al*., 2017).

Diverse types and concentrations of gelling agents have been used in different studies to investigate somatic embryogenesis, maturation of somatic embryos, biochemical characteristics (Klimaszewska *et al.*, 2000; Tsay *et al.*, 2006; Vahdati *et al.*, 2006; Seifeldin and Ozzambak, 2016), *in vitro* multiplication, proliferation and *ex vitro* rooting (Te-chato *et al.*, 2005; Masondo *et al.*, 2015; Lebedev *et al.*, 2019), however, the effect of the gelling agents on improvement of stomatal functionality of *in vitro* explants has not been studied so far, therefore, the aims of this study were to investigate the effect of two types of gelling agents (Agar and Gelrite) on the stomatal characteristics of walnut tissue culture explants to track their effects on water conservation capacity of *in vitro*-generated explants during *ex vitro* desiccation.

Material and Methods

Plant materials and growth conditions:

Micropropagated shoots of Persian walnut (cv. Chandler) in proliferation phase that were transferred every 3–4 weeks to the fresh medium were used in this study. At the last subculture, explants (nodal shoots, 20 ± 2 mm length), were cultured on the 60 mL of DKW medium (Driver and Kuniyuki, 1984) in jars with 65 mm diameter and 85 mm height supplemented with IBA (0.01 mg L^{-1}), BAP (1 mg L^{-1}) and sucrose (30 g L^{-1}). Two types of gelling agents including Agar and Gelrite at 7 and 2.1 g L^{-1} were used in DKW culture medium, respectively. The pH of the medium was fixed at 5.5 before autoclaving (for 20min at 121°C). Five containers were used for each treatment and two explants were cultured per container. The samples were incubated in a controlled chamber for 4 weeks at 25 ± 2 °C and 16-h photoperiod under 50 µmol m^{-2} s^{-1} irradiance. At the end of the experiment, walnut micropropagated plantlets were evaluated.

Morpho-physiological characteristics:

After removing the shoots from the culture medium at the end of experiment, vegetative and morpho-physiologic characteristics of plantlets were measured. The chlorophyll (Chl) index was recorded using a SPAD chlorophyll meter (SPAD, Konika Minolta, Japan), and shoot lengths were measured with a ruler.

Proline concentration was determined according to Bates *et al.* (1973). Free proline content in samples (µg g^{-1} DW) was calculated by equation {1} based on a standard curve drawn from five spectrophotometric readings by application a range of L-proline (0–200 µg mL^{-1}) as standard concentrations and the absorbance was read at 520 nm using toluene as the blank.

$$X = [(A.B)/C] /(D/5) \quad (1)$$

In this equation, X is µm proline/g of fresh weight material, A is µg proline/mL that obtained from the standard curve, B is ml toluene, C is the molar weight of proline and D is the g of the sample (Bates *et al.*, 1973).

Concentrations of glycine betaine (GB) in the leaf samples (µg GB g^{-1} leaf DW) were determined according to the method described by Grieve and Grattan (1983). The absorbance was measured at 365 nm with a spectrometer (Lambda 25- UV/VIS spectrometer) and reference standards of GB (range of concentration between 50–200 mg mL^{-1}) were prepared in 2 M sulfuric acid (Grieve and Grattan, 1983).

For determination of osmotic potential, leaves were cut into small segments, then placed in Eppendorf tubes perforated with some small holes and immediately frozen in liquid nitrogen. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at 15,000 g for 15 min at 4 °C. The collected supernatant was used for ψs determination. Osmolarity (c) was assessed with a vapour pressure osmometer (Osmomat 030-gonatec) and converted from mosmoles kg^{-1} to MPa

according to the Van't Hoff equation {2} (Martınez *et al.*, 2004).

$_s$(MPa) = -C (mosmoles kg^{-1}) × 2.58 × 10^{-3} {2}

Stomatal characteristics

To study the differences in stomatal morphology of the explant's leaves that were grown in a culture medium containing Agar and Gelrite, the epidermis of the underside of the second lateral leaflet (abaxial surface) was covered with a thin layer of transparent nail polish. After drying, the nail polish layer was removed from the epidermis by sticky tape and was mounted on microscopic slides. Stomatal traits examined using images of 300 stomata taken from these epidermal strips on the leaves of each treatment by using a light microscope (model Olympus). Finally, ImageJ (U.S. National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) was used to measure the stomatal length, stomatal width, pore length, pore width, the ratio of stomatal length to stomatal width, and stomatal density (Aliniaeifard and Van Meeteren, 2016).

Stomatal response to desiccation:

To measure the total transpiration rate (E) (stomatal and cuticular) of explant's leaves that were grown in media containing Agar and Gelrite, leaves were isolated from each of the explants and after measuring leaf area and initial fresh weight, were placed adaxial surface up on a balance and were gravimetrically weighed for a duration of 90 min at 5-min intervals under 50 mmol m^{-2} s^{-1} irradiance at room temperature and 50% RH, resulting in 1.24 kPa VPD (Aguilar *et al.*, 2000). Then the leaf samples were dried by an oven at 70 °C and the dry weight was recorded. The dynamic response of E {3} and relative water content (RWC) of the leaf samples was measured based on the methods described by Aliniaeifard *et al.* (2014) and Slavik (1974), respectively.

$$E = \{\{\left\{\frac{\Delta \text{ fresh weight (g)}}{\text{molar mass water } \left(\frac{g}{mol}\right)}\right\} \times 1000\left(\frac{\text{mmol}}{\text{mol}}\right)\} / \text{measurment frequency(s)}\}/\text{leaf area } (m^2)$$

Statistical analysis

In this experiment, GraphPad Prism 5 for Windows (GraphPad Software Inc., San Diego, CA) and SAS 9.2 were used for analyzing the data. For stomata morphological and morpho-physiological traits, data were subjected to unpaired T test. The difference among treatments means were compared by using Dancan's multiple range test $P \leq 0.05$. In this experiment, the data for transpiration rate (E) were fitted using two sigmoidal curves, non-linear regression with one-phase exponential decay, E = (E0 - Bottom)×exp(-K×X) + Bottom, where E0 is E at time zero, K is the slope of the curve and X is time and RWC were fitted using sigmoidal dose-response curve with a variable slope [E = Bottom + ((Top-Bottom)/(1 + 10(RWC50- RWC).Slope))]. F test was used for comparing the parameters of the fitted curves.

Results

Morpho-physiological characteristics of walnut explants that were grown in Agar and Gelrite media were analyzed. No significant changes in morphological and physiological characteristics including shoot length, chlorophyll content, leaf osmotic potential, and proline and glycine-betaine were detected as a result of using Agar and Gelrite in culture media (Table 1).

Table 1. Morpho-physiological characteristics of *in vitro* explants of Persian walnut *cv.* 'Chandler' grown in DKW medium containing Agar and Gelrite gelling agents

	Agar (Mean±SE)	Gelrite (Mean±SE)	DF	T value	P value
Shoot length (cm)	5.93±0.202	6.56±0.23	4	2.05	0.1098^{ns}
Chl content (SPAD)	27.85±1.37	27.06±0.97	4	0.47	0.6661^{ns}
Proline (µmol g^{-1} DM)	5.021±0.068	4.917±0.083	4	0.96	0.3908^{ns}
Glycine betaine (µmol g^{-1} DM)	1.527±0.034	1.44±0.016	4	1.90	0.1308^{ns}
Osmotic potential (MPa)	-1.255±0.027	-1.234±0.038	4	0.16	0.8800^{ns}

Data are mean values ± standard errors (SE). Degrees of freedom (DF), T and P value of treatment effects that were compared by unpaired t test ns: Non significance.

Although the use of Agar and Gelrite gelling agents in DKW culture media had no considerable effect on the morphological and physiological characteristics of walnut explants. Various stomata abnormalities were observed on walnut explants in the current study. Apart from stomatal width, pore length, and stomatal density, the stomatal length, pore width, and the ratio of stomatal length to stomatal width were significantly affected by the gelling agents in DKW medium. Stomatal length in explants that were grown in medium containing Gelrite was 12.29% longer than stomatal length in medium containing Agar. In contrast, the pore width was reduced by 33.39% using Gelrite in the culture medium, which is indicative of closed stomata in explants cultured in the medium containing Gelrite (Table 2).

Table 2. Stomatal traits of *in vitro* explants of Persian walnut *cv.* 'Chandler' grown in DKW medium containing Agar and Gelrite gelling agents

	Agar (Mean±SE)	Gelrite (Mean±SE)	DF	T value	P value
Stomatal length (µm)	24.43 ± 0.963^{b}	27.85 ± 0.952^{a}	8	2.525	0.0355^{*}
Stomatal width (µm)	22.01 ± 0.689	23.35 ± 0.680	8	1.386	0.2032^{ns}
Pore length (µm)	14.98 ± 0.748	14.98 ± 0.748	8	2.280	0.0521^{ns}
Pore width (µm)	6.924 ± 0.125^{a}	4.616 ± 0.160^{b}	8	5.677	0.0005^{**}
Stomatal length/width (µm)	1.112 ± 0.0181^{b}	1.196 ± 0.027^{a}	8	2.560	0.0336^{*}
Stomatal density (no.mm^{-2})	383.4 ± 22.56	333.6 ± 24.19	8	1.507	0.1703^{ns}

Data are mean values ± standard errors (SE). Degrees of freedom (DF), T and P value of treatment effects that were compared by unpaired t test. Ns Non significance. * Significance at 0.05 probability level. ** Significance at 0.01 probability level.

In explants grown in Agar-contained medium, round stomata were observed in comparison with more elliptical stomata shape in explants grown in Gelrite-contained medium. The ratio of stomatal length to stomatal width in explants grown in Gelrite-contained medium was about 7% higher than this ratio in explants grown in Agar-contained medium (Table 2). This is indicative of improving effect of Gelrite on the normal appearance of the stomata (Fig. 1)

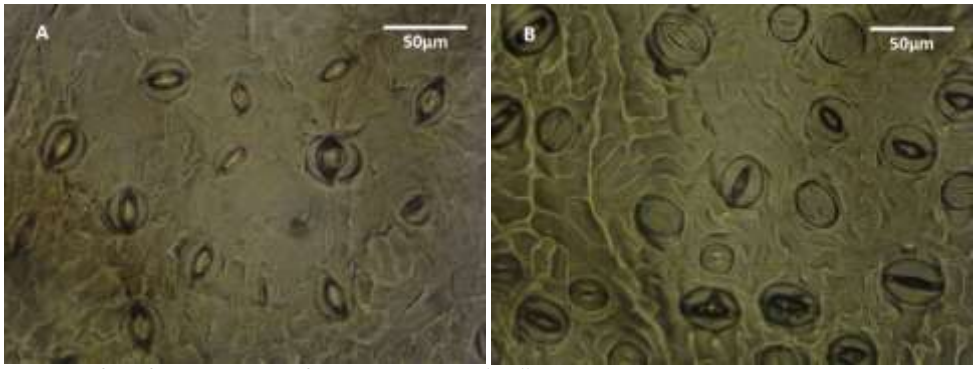

Fig. 1. Stomata on abaxial surface of *in vitro* explants of Persian walnut *cv.* 'Chandler' leaves grown in DKW medium containing Agar (A) and Gelrite (B) gelling agents

E and RWC during desiccation were considerably influenced by the type of gelling agent used in the walnut culture media. According to the obtained results, the use of Gelrite in DKW culture medium compared to the Agar, led to a decrease in E and increased the water conservation capacity of the explants (Fig. 2A and B). The amount of E during desiccation of explants grown in Gelrite-contained medium was about 31% lower than the amount of E of explants grown in Agar-contained medium (Table 3 Top). Leaves of explants grown in the medium containing Gelrite kept a higher E at the plateau part of the E × time curve (Table 3 Bottom), which can be related to the less development of cuticular layer on the leaf of Gelrite-grown explants.

Span (the distance between the top and bottom of the E × time curve) in explants grown in Gelrite-contained medium was significantly lower (about 38%) than the Span of the explants grown in Agar-contained medium (Table 3 Span).

There was a significant influence of Gelrite in DKW medium as gelling agents on water loss of leaves during desiccation, and leaf RWC curve was considerably different from leaf RWC of explants grown in Agar-contained medium (Fig. 2 B). Gelrite in DKW medium caused an increase in water conservation capacity as a result of improved stomatal morphology (Fig. 2). The Span between top and bottom of the RWC curve for explants grown in medium containing Gelrite was 35% lower than the span of the RWC curve for explants grown in Agar containing medium. Slope in response to desiccation for Agar-contained medium was 38% steeper in explants grown in Gelrite-contained medium. This indicates a decrease in the RWC of plants grown in Agar medium (Table 4 Slope), and walnut explants in Gelrite-contained medium showed better ability to conserve water compared to the explants in Agar-contained medium (Table 4 Bottom). As the consequence, bottom of the RWC curve in Gelrite-grown explants stayed in the upper levels than the RWC of Agar-grown explants. The relationship between E and RWC (E × RWC curve) also confirmed that leaves of Gelrite-grown explants have more capability to conserve their internal water in every given E (Fig. 2 C). For instance, in 80% RWC, leaves of Gelrite-grown explants had 1 mmol m^{-2} s^{-1} E, while Agar-grown explants exhibited 2 mmol m^{-2} s^{-1} E, indicating twice water loss in any internal RWC of the leaf. This would result in more susceptibility of Agar-grown explants to *ex vitro* desiccation.

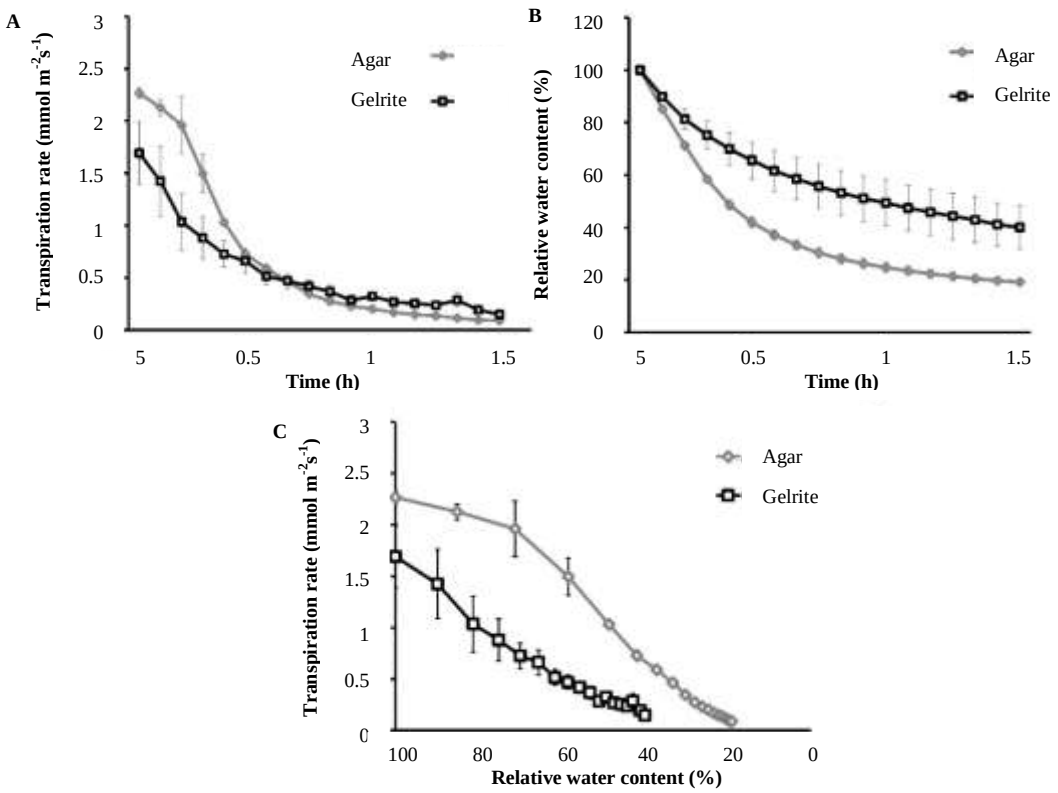

Fig. 2. Leaf transpiration rate (A), relative water content [RWC (B)] and transpiration rate as the function of RWC (C) of *in vitro* explants of Persian walnut *cv.* 'Chandler' grown in DKW medium containing Agar and Gelrite gelling agents

Table 3. Parameters of curve fitting for transpiration rate of *in vitro* explants of Persian walnut *cv.* 'Chandler' grown in DKW medium containing Agar and Gelrite gelling agents

	Agar	Gelrite
Top	3.099±0.134[a]	2.123±0.185[b]
Bottom	-0.049±0.065[a]	0.1922±0.064[b]
Span	3.149±0.11[a]	1.931±0.16[b]

Table 4. Parameters of curve fitting for relative water content (RWC) of *in vitro* walnut explants (cv. Chandler) grown in DKWmedium containing Agar and Gelrite gelling agents

	Agar	Gelrite
Bottom	18.36±0.76[a]	36.46±6.56[b]
Slope	2.91±0.10[a]	1.79±0.52[b]
Span	105.9±1.84[a]	72.21±6.27[b]

Discussion

In the present study, due to the influence of the gelling agents on morpho-physiological characteristics of tissue culture explants through affecting the osmotic potential of different culture media, morpho-physiological characteristics of walnut explants that were grown in Agar and Gelrite media were measured. No significant changes in morphological and physiological characteristics were observed as a result of using Agar and Gelrite in the culture media (Table 1). Based on our primary assumption that when Agar is chelated absorbs nutrient ions and delays plant growth due to the unavailability of essential nutrients in the culture media (Debergh, 1983; Masondo *et al*., 2015), accordingly, the growth rate of shoots grown in medium containing Gelrite was higher than medium containing Agar. Based on the results of previous studies, compared to Agar, use

of phytagel in the culture medium improves plantlets regeneration, average stem length, and quality of grown shoots (Te-chato *et al*., 2005; Kacar *et al*., 2010; Masondo *et al*., 2015). It is generally accepted that the mineral composition of the gelling agents and the interaction between the gelling agents of the culture medium with the plant, plays an important role in the growth of *in vitro*-cultured plants (Seifeldin and Ozzambak, 2016; Eshghi Khas *et al*., 2020).

Although the use of Agar and Gelrite gelling agents in DKW culture media had no considerable effect on the morphological and physiological characteristics of walnut tissue cultured explants, significant differences were detected in some of the stomatal characteristics (Table 2). Our previous investigations on micropropagated walnut plantlets confirmed that stomatal malfunctioning is the main reasons for the uncontrolled water loss during transfer to *ex vitro* condition and lack of success in the acclimatization of walnut plantlets (Hassankhah *et al*., 2014; Asayesh *et al*., 2017a; Asayesh *et al*., 2017b; Vahdati *et al*., 2017).Van den Dries *et al*. (2013) have reported that hyperhydricity is the other factor that leads to occurrence of abnormalities and stomatal dysfunctioning with reduced capability of normal closing responses (Van den Dries *et al*., 2013). However, various reasons for these abnormalities that lead to wilting of micropropagated plants have been also reported, one of the most important of which is high relative humidity (RH) of the culture media (Hazarika, 2006; Koubouris and Vasilakakis, 2006; Asayesh *et al*., 2017b). Many plant species lose their stomatal function by either being exposed to high relative humidity during leaf expansion (Fanourakis *et al*., 2020) or when fully developed leaves are exposed to high air humidity (Aliniaeifard and van Meeteren, 2014; Aliniaeifard *et al*., 2014; Aliniaeifard and van Meeteren, 2016). Although, it has been reported that the effect of the gelling agent types on the stomatal morphology is more through their effect on the osmotic potential or through hyperhydricity, in the current study, hyperhydricity was not observed in walnut tissue culture explants.

In Agar-contained medium, round stomata were observed in comparison with more elliptical stomata shape in explants grown in Gelrite contained medium. This is indicative of improving effect of Gelrite on the normal appearance of the stomata (Fig. 1). In agreement with our results, Van den Dries *et al*. (2013) showed that stomata are partially or fully closed in the hyperhydric leaves that caused by using Gelrite as gelling agent, in *Arabidopsis thaliana* Stomatal density did not show any significant difference between Agar- or Gelrite-grown explants. The formation of big size of stomata with larger pores as a result of high RH is one of the common responses of plants during leaf development. The changes in stomatal size are generally more prominent in stomatal length, as compared to stomatal width, but the effect of high RH on stomatal density is strongly species dependent (Fanourakis *et al*., 2013; Aliniaeifard *et al*., 2014; Fanourakis *et al*., 2020). Accumulation of water and lack of oxygen in the apoplast have been reported in seedlings emerged from Gelrite, which resulted in the development of hyperhydricity symptoms (Van den Dries *et al*., 2013). The pore aperture in those plants was reduced and the stomata stayed more closed, which could be the reason for better adaptation capacity during transplantation for the seedlings with a little hyperhydricity. In general, the type and concentration of gelling agent used in the culture medium affect the accessibility to the water. It has been confirmed that impact of Gelrite on the occurrence of hyperhydricity is due to its effects on water availability through improving water uptake (Franck *et al*., 2004; Ivanova and Van Staden, 2011; Pourkhaloee and Khosh-Khui, 2015). However, Apostolo and Llorente (2000) showed that hyperhydric seedlings have basic anatomical abnormalities that prevent them from surviving during acclimatization (Apostolo and Llorente, 2000).

For micropropagation plants, the desiccation and wilting of newly transferred plantlets are a result of uncontrolled

water loss from plantlets. Therefore, by the use of diverse approaches, it has been tried to increase the leaf water conservation capacity of *in vitro* plants. Methods such as the reduction of relative humidity in the headspace of culture vessels, higher irradiance and CO_2 concentration, the use of anti-transpirants like ABA and decrease in osmotic potential of the culture medium by PEG have been reported in previous studies (Shim *et al*., 2003; Cha-um *et al*., 2010; Asayesh *et al*., 2017b; Vahdati *et al*., 2017; Aliniaeifard *et al*., 2020). Although there is no documented information on the role of the type of gelling agent and its possible effects on the improvement of stomatal morphology and functioning in the *in vitro* condition, the results of the present study showed the effect of Gelrite gelling agent on stomatal morphology and reduction of transpiration rate during desiccation of walnut tissue culture plantlets. Previous studies determined that the appropriate culture medium for proliferation of Persian walnut is DKW medium containing 2.2 gL-1 of phytagel or Gelrite (Driver and Kuniyuki, 1984; Saadat and Hennerty, 2002; Vahdati *et al*., 2004 and 2006). Therefore, Gelrite is more commonly used for DKW medium than Agar.

In the present study, walnut tissue cultured explants did not show signs of hyperhydricity, but the improvement of stomatal characteristics and consequently decreased transpiration rate and increased water conservation in response to desiccation were observed as a result of Gelrite application in the DKW culture medium. Improvement of *ex vitro* desiccation as a result of improvement of stomatal functionality has been previously reported by decrease in relative humidity (Asayesh *et al*., 2017b), CO_2 enrichment (Vahdati *et al*., 2017) and other stomata closing stimuli (Aliniaeifard *et al*., 2020) in the walnut culture medium. Here, we showed that Gelrite can be another option for promoting stomatal functionality and as a consequence improvement of desiccation tolerance in *in vitro* walnut generated plantlets.

Conclusions

In the present study, the use of Gelrite as a gelling agent in comparison with Agar, improved stomatal functionality, which led to a reduction in E during desiccation and increased RWC and walnut explants capacity to conserve its water content, which can help explants to cope with desiccation in *in vitro*-generated explants of Persian walnut when faced to unfavorable *ex vitro* conditions.

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