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Improved control of water loss from micropropagated grapevines (*Vitis vinifera* cv. Nebbiolo)

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Summary

Micropropagated plantlets are generally susceptible to rapid desiccation when exposed to reduced relative humidity and require a costly acclimatization procedure. Detached leaves of micropropagated *Vitis vinifera*, cv. Nebbiolo, plantlets were used to evaluate the relative contribution of leaf cuticle and stomata to water loss. Water loss occurred mainly from the abaxial surface of detached leaves; moreover, a large majority of stomata was still open 3 h after exposure to 63 % RH. An indirect estimation of epicuticular wax suggested a lower wax deposition for micropropagated plantlets compared to acclimatized and field-grown plants of the same clone. A previously developed method to produce hardened micropropagated plants was adopted: 1 mg·l^{-1} paclobutrazol (PBZ) was added to the medium and culture vessels with reduced relative humidity (rRH) were used during the last stage of micropropagation. Under our experimental conditions, rRH was more effective to reduce transpiration than PBZ; a combination of both treatments improved plant survival during acclimatization.

K e y w o r d s : grape, micropropagation, acclimatization, hardening, stomata, PBZ.

A b b r e v i a t i o n s : MS, MURASHIGE and SKOOG 1962; PBZ, paclobutrazol, N-dimethylaminosuccinamic acid; RH, relative humidity; hRH, high RH; rRH, reduced RH.

Introduction

The *in vitro* environment is known to induce modifications of morphological, anatomical and physiological features of micropropagated plantlets. Sealing culture vessels leads to a water-saturated atmosphere, and plants grown in that atmosphere are generally susceptible to rapid desiccation when exposed to lower relative humidity. Water is lost through the leaf cuticle due to the lack of well developed epicuticular wax, and through stomata because they do not close normally in response to water stress (review: PREECE and SUTTER 1991). The relative contribution of stomata and cuticle to water loss of micropropagated plants during acclimatization is not well understood and may depend on the species and the culture conditions.

The cost of the acclimatization stage is generally considerable. Procedures leading to an *in vitro* hardening of shoots or plantlets during the last micropropagation phase allow an easier acclimatization, with lower costs of labor and equipment. A method to produce more hardened micropropagated plants was previously developed: roots are protected by cellulose plugs (ROBERTS and SMITH 1990), and PBZ or other growth retardants are added to the culture medium (SMITH *et al*. 1990 a, 1991; ROBERTS and MATTHEWS 1995); moreover, the use of culture vessels with rRH is recommended (SMITH *et al*. 1990 b). The method was successfully applied to chrysanthemum, rose and grapevine (SMITH *et al*. 1992), which acclimatized more rapidly or even did not need acclimatization if PBZ and rRH were applied during stage III of micro-propagation.

In the present investigation, grapevine plantlets were grown *in vitro* after addition of PBZ to the liquid culture medium and/or by reducing RH inside the culture vessels. The experiment allowed to study the relative contribution of stomata and cuticle to water loss of leaves taken from micropropagated plantlets and the effect of PBZ and rRH on deposition of epicuticular wax and stomatal efficiency to reduce transpiration.

Material and Methods

Grapevine (*Vitis vinifera* L. cv. Nebbiolo, clone CVT36) mother plants were cultivated on solid medium in glass jars (750 ml) with glass lids. The medium consisted of MURASHIGE and SKOOG (1962) (MS) mineral salts, MS vitamins, 20 g·l^{-1} sucrose; no plant growth regulators were added. The pH was adjusted to 5.6 before the addition of agar (8 g·l^{-1}) and autoclaving at 120 °C for 10 min.

Individual shoots (length: 30-40 mm) were excised from mother plants and placed in cylindrical cellulose plugs (Sorbarods, Baumgartner Papiers SA, Switzerland) soaked with liquid medium. The medium was identical to the modified MS described above, except for the exclusion of agar, and the addition of 1 mg·l^{-1} PBZ (paclobutrazol, a triazole growth retardant) in half of the treatments. The plugs were placed in clear polystyrene vessels (60 plugs per vessel) which had been sterilized by gamma irradiation. Half of the vessels had lids with four 20-mm diameter holes drilled along both flanks and overlaid by strips of Tyvek, a selectively permeable membrane (E.I. du Pont de Nemours & Co., U.S.A.). Vessels with Tyvek-covered holes maintained rRH at 94 % while in vessels with intact lids RH was 100 % (hRH; SMITH *et al.* 1990 b). Effects of PBZ and of rRH were assessed in a factorial scheme with 60 replicates per combination.

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All cultures were maintained in a culture room at 25 °C, in a 16-h photoperiod provided by fluorescent lamps (L58 CoolWhite, Osram, Germany) providing 45 μmol·m^{-2}·s^{-1}. Various parameters were measured after 4 weeks.

Field- and greenhouse-grown grapes have hypostomatal leaves (DÜRING 1980). Our preliminary microscopic observations on leaves from micropropagated grapes confirmed that stomata were largely confined to the abaxial leaf surface. Three protocols for preparation of detached leaves were followed: No modification (control leaves, Treatment A); to prevent evaporation from the majority of stomata, a petrolatum layer was applied by a syringe to the abaxial surface of leaves (Treatment B); to prevent transpiration from the adaxial leaf surface and to evaluate stomatal water loss, petrolatum was applied to the adaxial surface (Treatment C). Leaf water loss (in % of the initial fresh weight) was determined 30, 60, 120, 160 and 200 min after exposure to 60 % RH at 22 °C. Five leaves were used per treatment.

The stomatal density (number of stomata·mm^{-2}), the stomatal index [number of stomata/(number of stomata + number of epidermal cells)], and the stomatal length:width ratio were assessed by epidermal impressions: transparent nail varnish was applied to the abaxial surface of leaves and the epidermal imprints were investigated microscopically. Twelve leaves per treatment, using the 2nd fully developed leaf from the apex, were measured; three observations per leaf (stomatal density and index) or measurements of the length:width ratio of 100 stomata were averaged. The percentage of completely closed stomata was determined for leaves that were maintained in darkness for 60, 120 and 180 min at 63 % RH and 22 °C. Stomatal closure was assessed using nail varnish impressions of 50 stomata on two leaves per treatment, taken immediately after opening of the culture vessels.

The presence of epicuticular wax on the leaf adaxial surface was evaluated indirectly by using a Contact-angle Meter (mod. G1, Krüss, USA: dropping method, static contact angle). The contact angle of a small ultrapure-water droplet with the leaf upper surface was measured through an optical system. The same test was performed on leaves from field-grown plants of the same clone and from micropropagated plantlets (treatment -PBZ/hRH) after acclimatization. In the case of acclimatized plantlets, *in vitro*- and *in vivo*-formed leaves were examined separately. Measurements were made on 20 leaves per treatment, averaging two measurements per leaf.

For chlorophyll estimation, single leaves were weighed and placed in 3 ml methanol and treated as described by ROBERTS *et al*. (1990). The results (average of 12 replications per treatment) were expressed as μg of total chlorophyll mg^{-1} of fresh weight.

Results

The relative contribution of stomata and cuticle to water loss of micropropagated grape plants during acclimatization is shown in Fig. 1. In intact leaves, after more than 3 h at 63 % RH a substantial water loss was registered for all the leaves, but it was significantly lower if plantlets were previously cultivated at reduced RH. Comparing treatments B and C, in which a petrolatum layer was applied to the adaxial or the abaxial leaf surface, the majority of water was lost through the abaxial surface likely because of an imperfect stomatal closure. If the leaf upper surface was petrolatum-coated (treatment C), plantlets grown at rRH were less dehydrated. In no case PBZ in the medium had a significant effect.

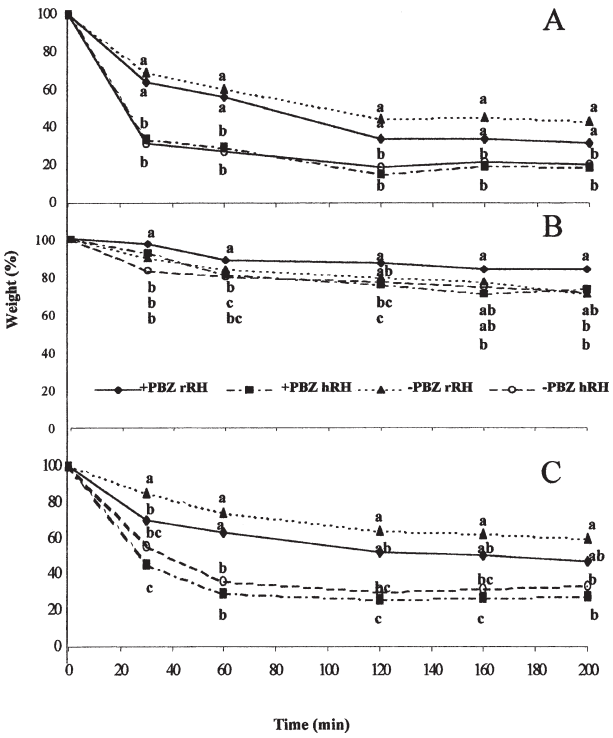

Fig. 1: Water loss (% of the initial fresh weight) from detached leaves of cv. Nebbiolo subjected to different treatments: control leaves (**A**); a layer of petrolatum applied to the abaxial leaf surface (**B**) or to the adaxial leaf surface (**C**). Leaf water loss was measured during exposure to 60 % RH. For each measurement, different letters indicate significant differences according to the Duncan's test, P = 0.05.

Both PBZ and RH significantly influenced stomatal density, in an opposite way (Tab. 1): plantlets treated with PBZ had a higher number of stomata·mm^{-2} compared to untreated plantlets, while after rRH treatments the stomatal density was lower. No difference was found among treatments as for stomatal index and stomatal length:width ratio. Adult leaves of field-grown Nebbiolo CVT36 vines had a lower stomatal density (165 stomata·mm^{-2}) and more elongated stomata (length:width ratio = 1.43) compared to leaves of *in vitro*-cultivated plants.

If plantlets were exposed to an environment leading to stomatal closure, stomata closed more efficiently if RH inside the vessels was reduced during the last period of *in vitro* culture (Fig. 2). The presence of PBZ in the culture medium did not influence significantly the percentage of completely closed stomata, but the mean aperture of open stomata was anyway reduced (data not shown).

Both PBZ and rRH increased the contact angle of a water droplet on the surface of the leaf (Tab. 2). This parameter expresses the wettability of the surface and is linked to the presence of wax or similar compounds at the tested surface,

Table 1

Stomatal density, stomatal index and stomatal length:width ratio of *in vitro*-grown leaves at high or reduced relative humidity (hRH, rRH), with or without paclobutrazol (PBZ). Measurements were made on 12 leaves per treatment, averaging 3 observations per leaf. **: significant at P = 0.01; n.s.: not significant

	Stomatal density (stomata·mm^{-2})		Stomatal index		Stomatal length:width ratio	
	rRH	hRH	rRH	hRH	rRH	hRH
+ PBZ	360	438	0.123	0.100	1.27	1.31
- PBZ	205	329	0.102	0.113	1.31	1.24
PBZ	**		n.s.		n.s.	
RH	**		n.s.		n.s.	
interaction	n.s.		n.s.		n.s.	

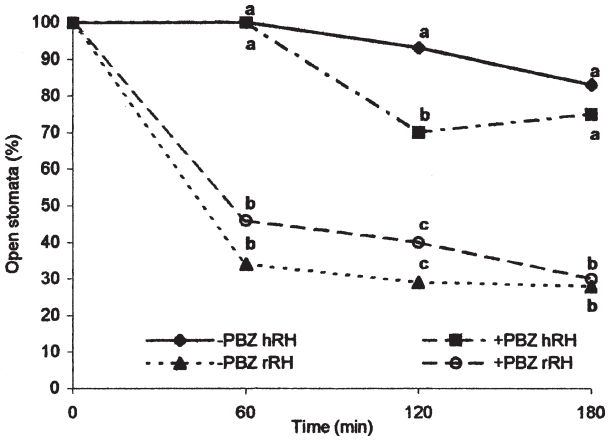

Fig. 2: Percentages of open stomata of leaves which were detached and exposed to 63 % RH in the darkness for up to 3 h. For details see Tab. 1.

thus giving an indirect indication for the presence of epicuticular wax. Previous attempts to extract wax and measure it gravimetrically or colorimetrically failed presumably due to the low amount of wax. After acclimatization of micropropagated plantlets (treatment -PBZ/hRH), the average value of the contact angle was 59.1° for *in vitro*-formed leaves, 68.0° for *in vivo*-formed leaves. The average angle for leaves collected in the field was 104.9°.

There was an increase in the amount of chlorophyll·mg^{-1} of leaf fresh weight in plantlets cultured at rRH, while this parameter was not influenced in the presence of PBZ (Tab. 2).

Discussion

The water loss of micropropagated plants during acclimatization has been primarily attributed to stomatal malfunctioning and to a reduced deposition of epicuticular wax on the leaf surface. The relative contribution of these two factors has been discussed controversially. Many authors reported that stomata of micropropagated plantlets are abnormal in morphology and density and are unable to close (Conner and Conner 1984; Santamaria and Kerstiens 1994),

Table 2

Contact angles of water droplets with adaxial leaf surfaces and total chlorophyll content of leaves. Measurements were made on 20 leaves per treatment averaging two measurements per leaf (contact angle), or 12 replications per treatment (chlorophyll content). **, *, n.s.: significant at P = 0.01, P = 0.05 and not significant, respectively. For details see Tab. 1

	Contact angle (degree)		Total chlorophyll (µg·mg^{-1} fresh weight)	
	rRH	hRH	rRH	hRH
+ PBZ	65.5	62.2	2.95	1.62
- PBZ	58.5	52.2	2.56	1.63
PBZ	**		n.s.	
RH	*		**	
interaction	n.s.		n.s.	

while according to others (Shackel *et al.* 1990) stomata can be functional. It was observed that the amount of leaf epicuticular wax was low (Grout 1975), abnormally structured (Sutter and Langhans 1982), and chemically different from wax of greenhouse-grown plants (Sutter 1984). For grape, Iacono and Martinelli (1998) reported that cuticular transpiration of *in vitro*-grown plantlets might be relevant but that it is strongly influenced by the genotype. To our knowledge, there is still no reliable method to accurately determine cuticular transpiration. According to Düring and Harst (1996) stomata of *in vitro* grapes are functional to a certain extent but do not close completely in the dark. Fila *et al.* (1998) observed that stomata of micropropagated grapes are functional in response to light and CO_2 but leaf conductance responds only slightly to changes in vapour pressure deficit. Those different behaviours could be attributable not only to the different genotypes but also to different culture conditions and materials (epidermal strips, detached leaves, shoots, and whole plants). Moreover, the plant water balance also depends on the ability of the plant to uptake and transport water. Novello *et al.* (1992 a and b) found that in micropropagated grapes roots play an active role in replacing water loss.

In our experiment, grapes cultivated on PBZ-free medium and in sealed vessels (-PBZ/hRH) can be considered as plantlets grown under common *in vitro* conditions. Leaves detached from these plantlets lost water mainly from the abaxial surface, and their imperfect stomatal functioning was confirmed assessing stomatal closure after exposure to lower RH. The indirect estimation of epicuticular wax also suggested a lower wax deposition of *in vitro* plants compared to acclimatized and field-grown vines.

Previous experiences on the transfer of micropropagated plantlets to soil proved that wilting was significantly reduced if the growth retardant PBZ was included in the medium and the RH in culture vessels was lowered (Smith *et al.* 1990a and b, 1992). In chrysanthemum both treatments improved stomatal activity and PBZ induced a higher deposition of epicuticular wax.

In the present investigation both PBZ (1 mg·l^{-1}) and reduced RH (94 %) increased the contact angle between the leaf surface and a water droplet, suggesting an increased

deposition of epicuticular wax (GROUT and ASTON 1977), but we were not able to determine the amount of wax quantitatively.

When the abaxial leaf surface was coated with petrolatum, no significant difference could be detected among treatments and leaf dehydration through the cuticle of the adaxial surface was relatively low. When the upper surface was coated, leaves formed at rRH lost less water. After exposure of detached leaves to a lower RH, stomatal closure of leaves formed at rRH showed significantly higher percentage and occurred more rapidly compared to leaves formed at hRH; this confirms their better functioning.

In a previous experiment with grape, SMITH *et al*. (1992) exposed detached leaves to 20 % RH: with PBZ in the culture medium the average stomatal aperture was reduced, while the stomata closed completely if the leaves were formed at rRH. Under our experimental conditions, PBZ was not as much effective as rRH in improving stomatal efficiency. PBZ increased the number of stomata·mm^{-2} while rRH had an opposite effect; the higher stomatal density can contribute to explain the minor effect of PBZ on the control of water stress. The effect of reduced RH on stomatal density and index is still controversial: in proliferating plum shoots, SCIUTI and MORINI (1993) observed a decrease in stomatal density and index if RH was lowered. CAPELLADES *et al.* (1990) observed an opposite effect with micropropagated rose shoots. In cv. Valiant grape plantlets the addition of PEG to the culture medium to induce water stress allowed a higher survival rate but stomatal index was unaffected, suggesting that stomatal physiology would be more relevant (DAMI and HUGHES 1997).

PBZ and rRH also proved to increase root number and thickness (SMITH *et al.* 1992): in whole plants this may facilitate the replacement of water loss from leaves. The positive effect of rRH on the amount of chlorophyll may enhance the rate of photosynthesis of plantlets.

Under the conditions of our experiments a reduction of RH appears to have a major effect on grape by enhancing stomatal functioning, thus allowing a better control of water loss from leaves. These results may help to understand how micropropagated grapes lose water during acclimatization, and how PBZ and rRH improve this process by modifying morpho-physiological features.

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