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Relationships Between Rooting Ability and Changes of Endogenous IAA and ABA During the Rooting of Hardwood Cuttings of some Grapevine Rootstocks

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Abstract

In this study, relationships between rooting capability and the changes of endogenous IAA and ABA hormone levels during rooting of hardwood cuttings of 5 BB, 41 B, and 420 A grapevine rootstocks were studied by RP-HPLC. The chromatographic method that we developed was used for the separation and determination of these hormones. For this purpose, the mobile phase composition was optimised in order to separate IAA (indole-3-acetic acid) and ABA (abscisic acid). Mobile phase was optimised by establishing relationships between retention parameters and single solvent polarity parameter. pH measurements in acetonitrile-water binary mixtures were carried out by using IUPAC rules. Different levels of IAA and ABA substances correspond to the different rooting ability of 5 BB, 41 B and 420 A hardwood cuttings. Low IAA and high ABA levels were detected in cuttings of 41 B and 420 A having low rooting rate, whereas high IAA and low ABA levels were detected in cuttings of 5 BB, having high rooting rate in dormant stage. In general, endogenous IAA increased, while ABA decreased during rooting process. It was found that there was a positive correlation between IAA level and rooting rate, but a negative correlation between ABA level and rooting rate.

Zusammenfassung

Zusammenhang zwischen Bewurzelungsfähigkeit und endogenem IAA- und ABA-Gehalt bei Steckhölzern von Rebenwurzelstöcken. In dieser Untersuchung wurden die Zusammenhänge zwischen der Bewurzelungsfähigkeit und dem endogenen IAA- und ABA-Hormongehalt während der Wurzelbildung von Steckhölzern von 5 BB, 41 B, und 420 A Rebenwurzelstöcken mittels RP-HPLC ermitteln. Das hierfür entwickelte Chromatographieverfahren diente der Trennung und Bestimmung dieser Hormone. Für diesen Zweck wurde die Zusammensetzung der mobilen Phase für die Trennung von IAA (Indolessigsäure) und ABA (Abscisinsäure) optimiert. Diese Optimierung erfolgte dadurch, dass Beziehungen zwischen Retentionsparametern und den Polaritätsparametern von Lösungsmitteln hergestellt wurden. Die pH-Messungen in der Acetonitril-Wasser-Mischung wurde nach IUPAC-Richtlinien durchgeführt. Die verschiedenen IAA- und ABA-Konzentrationen entsprechen den Unterschieden in der Bewurzelungsfähigkeit der 5 BB-, 41 B- und 420 A-Steckhölzer. Geringe IAA- und hohe ABA-Konzentrationen fanden sich in 41 B- und 420 A-Steckhölzern mit geringer Bewurzelungsrate, wohingegen hohe IAA- und geringe ABA-Gehalte in 5 BB-Steckhölzern festgestellt wurden, die während der Ruhephase eine hohe Bewurzelungsfähigkeit aufweisen. Im allgemeinen nimmt der endogene IAA-Gehalt in der Bewurzelungsphase zu, während der ABA-Gehalt abnimmt. Zwischen IAA-Gehalt und Bewurzelungsrate konnte eine positive zwischen ABA-Gehalt und Bewurzelungsrate eine negative Korrelation festgestellt werden.

Key words. ABA – cuttings – IAA – plant hormones – rooting – RP-HPLC.

Introduction

Because of their inherent resistance to certain destructive insect pests, especially grape phylloxera (*Dactylosphaera vitifoliae*) and nematodes, American species have often proved valuable where own-rooted vines have failed. Rootstocks are also essential for the control of nematodes. Grapevine is propagated commercially by cuttings, buds, or grafted (WEAVER 1976).

The rooting of cuttings is a complex phenomenon which involves very different events. Rooting of grapevine rootstock hardwood cuttings involves a sequence of morphogenetic steps, each of which has different requirements of endogenous growth regulators (FUJII and NAKANO 1974; NORCINI et al. 1985; GASPAR et al. 1990; NORDSTRÖM and ELIASSON 1991; GARRIDO et al. 1998). Adventitious rooting depends on numerous endogenous factors among which growth substances such as auxin and abscisic acid (ABA) are believed to play a major role. Moreover, auxin seems to be an universal inducer of adventitious roots. Several reports have indicated a positive correlation between

endogenous indole-3-acetic acid (IAA) levels in cuttings and the number of adventitious roots produced per cutting (ALVAREZ et al. 1989; LIU et al. 1998). In general, the levels of these substances, as well as their balance, can have different effects on root formation (SMITH and THORPE 1975; KRACKE et al. 1981), but the phenomena involved have not been defined exactly. Morever, there have been far fewer investigations on endogenous hormonal changes during rooting of grapevine rootstocks.

The aim of the present work was to study relationships between the rooting capability and the changes of endogenous IAA and ABA hormones amount during rooting of hardwood cuttings of 5 BB, 41 B, and 420 A grapevine rootstocks. These hormones are present in plant material at very low concentrations ($ng\,g^{-1}$), together with many other compounds. Recently reversed phase high performance liquid chromatography (RP-HPLC) is the most commonly used method for the separation and determination of these compounds (DURLEY et al. 1982; WURST et al. 1984; MAZUR et al. 1997; MATTIVI et al. 1999; HOENICKE et al. 2001). There is no optimisation study for the chromatographic separation of IAA and ABA. In this study, the composition and the pH of the mobile phase were optimised in order to separate indole-3-acetic acid (IAA) and abscisic acid (ABA). The mobile phase composition was optimised by establishing relationships between retention parameters and E_T^N (MICHELS and DORSEY 1988; REICHARDT 1988; BERGES et al. 2000). This relationship can be expressed as

$$\log k = e\,(E_T^N) + C$$

IAA and ABA are weakly acidic substance. The pH of the mobile phase is very important for optimizing the chromatographic separation for acidic compounds. In this study pH measurements in acetonitrile-water binary mixtures were carried out by using IUPAC rules (RONDININI et al. 1987; SCHOENMAKERS et al. 1991; BARBOSA et al. 1994; ROSES et al. 1996; BERGES et al. 2000).

Materials and Methods

Plant Material

Hardwood cuttings of 5 BB (*Vitis berlandieri* x *Vitis riparia*), 41 B (Chasselas x *Vitis berlandieri*) and 420 A (*Vitis berlandieri* x *Vitis riparia*), a very important rootstock for grape varieties, were used as plant material. 5 BB is an easy-to-root, 41 B and 420 A are relatively difficult-to-root rootstocks. The samples were taken from well matured canes during dormant stage. Cuttings were taken in mid-February from the median part of the canes (between the 5th and 15th node from the base). Cuttings had four buds; diameter was 8–9 mm. Later cuttings were planted in perlite substrate in a bottom unheated mist propagation unit. The experiment was established according to the randomised design with four replication and 30 cuttings in each replication. The data were analysed statistically and the differences were evaluated according to Duncan's Multiple Range Test.

To determine hormonal changes, cuttings were taken at five subsequent stages: 1) dormant bud, 2) swollen bud, 3) bud burst of the terminal bud, 4) root emergence and 5) rooted (roots 3–5 cm). These stages corresponded to 0, 10, 20, 30 and 45 days after the cuttings were planted, respectively.

Extraction

Ten grams of fresh tissue per sample were homogenised with 70 % (v/v) methanol and stirred overnight at 4 °C. The extract was filtered through a Whatman filter and the methanol evaporated in vacuum. The aqueous phase was adjusted to pH 8.5 with 0.1 M phosphate buffer and then was shaken three times with ethyl acetate. After removal of the ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5 with 1 N HCl. The solution was shaken three times with diethyl ether, and then was passed through waterless sodium sulphate. After diethyl ether phase was evaporated in vacuum, the dry residue containing hormones was dissolved in 2 ml methanol and stored in vials at 4 °C (DURLEY et al. 1982, WURST et al. 1984).

Chromatographic procedure

The chromatographic analysis was performed with a Shimadzu Model LC. The chromatographic system consists of a Shimadzu Model LC 10 ADvp pump with an auto injector (SIL 10 AD VP) and diode array detector and fluorescence detector (SPDM 10 A DAD; operating in the range of 200–400 nm; excitation wavelength 225 nm, emission wavelength 360 nm). This equipment has column oven (CTO 10 AVP) and degasser system (DGU 14 A). Separations were performed on a Luna C_{18}, 5 mμ particle size, 4.6 mm I.D x 250 mm (Phenomonex).

Throughout this study, the mobile phases assayed were acetonitrile-buffer (30:70; 35:65, v/v). In this media, 30 mm phosphoric acid adjusted to pH value 3.0 with sodium hydroxide, was used as the buffer component. The Luna C_{18} column was equilibrated for each mobile phase conditions for 30 min. The column temperature was maintained constant at 25 °C. The separation was carried out by isocratic elution with a flow rate of 0,8 ml min^{-1}. An injection volume of 20 μl was used for each analysis.

The standard of the individual acid was prepared in the mobile phase and chromatographed separately in order to determine the retention time for each acid. The signal of the compounds was monitored at 265 nm and 280 nm for ABA and IAA, respectively. The fluorescence excitation was set to 225 nm and emission was set to 360 nm for the detection of IAA. Capacity factors were calculated from $k=(t_R-t_o)\,t_o^{-1}$, where t_o was the retention time, and t_R the retention time of each hormone for each mobile phase. In this equation the retention time, t_o, was established for every mobile phase composition using potassium bromide solution [0,01 % (w/v) in water, $\lambda_{max}=200$ nm]. The retention times and the capacity factors of the solutes were determined from three different injections at each mobile phase composition considered. Peak identification was based on retention time and spiking of the sample.

Development of the Method

In this study, a reversed phase ion suppression HPLC method was used for the chromatographic separation of ABA and IAA. The mobile phase was adjusted to dif-

ferent pH in order to select a suitable pH condition. The results show that pH 3,0 is appropriate. The mobile phase was optimised by establishing relationships between retention parameters and the REICHARDT's E_T^N scale of solvent polarity for the chromatographic separation of abscisic acid and indole-3-acetic acid. Retention times and capacity factors are given in Table 1.

Table 1. Retention times and capacity factors*.

Compounds	% 30 ACN		% 35 ACN	
	$t_{r,ort}$	k	$t_{r,ort}$	k
IAA	18.4	4.35	10.9	2.23
ABA	21.0	5.10	11.8	2.50
KBr	3.44		3.37	

* pH 3.0; Flow rate: 0.8 ml min^{-1}.

The experimental log k values related with the E_T^N solvent parameter are given in Table 2. The multiparameter α, β and π* scale and the relationships with log k, have been applied to the prediction of the chromatographic behaviour of plant hormone substances (Table 2).

From Table 2 it can be observed that IAA and ABA showed a similar elution profile. Log k and E_T^N correlate linearly over the whole experimental range of acetonitrile contents studied. The little differences between slopes indicate that good chromatographic separation can be obtained for the plant hormones when the acetonitrile content in the mobile phase is 30% (v/v), and the separation factor values are appropriate. In this condition the α values are higher.

Using this mobile phase the desired acids were clearly separated. Typical chromatograms for these acids in plant material are shown in Fig. 1 and Fig. 2.

Quantification by external standard method was carried out using peak areas. Table 3 shows the performance characteristics of the method.

It can be seen from Table 3 that the precision of the method is sufficient. A linearity has been obtained for each acid inside in the range studied with a correlation coefficient higher than 0.999. The recovery for plant tissue extracts spiked with ABA and IAA were found to be 110% and 108%. The optimisation of the chromatographic conditions leads to a chromatogram in which the two plant hormones are well resolved.

Results

Rooting Rates

Results of rooting of cuttings are shown in Table 4. It was found that rooting rates were 62.7%, 47.4%, and 45.7% in 5 BB, 41 B, and 420 A rootstocks, respectively.

Table 4. Rooting rates of cuttings of rootstocks.

Rootstocks	Rooting Rate (%)
5 BB	62.7 a[1]
41 B	47.4 b
420 A	45.7 b

[1] Duncan's multiple range test, $p<0.05$.

Table 2. Relationships between log k for IAA and ABA and E_T^N and α, β, π* solvatochromic parameters of the eluent system in the interval studied using the LSER approach.

Compounds	Log k–E_T^N equation	Multiparameter equation
IAA	log k=18.0E_T^N – 15.0	Log k=5.28 – 2.81α – 14.0β + 2.92π*
ABA	log k=19.3E_T^N – 16.0	Log k=3.93 – 2.06α – 12.5β + 3.06π*

Table 3. Performance characteristics of the method*.

Hormone	LR	Regression Equation	S_{xo}	CV	N
ABA	Up to 5.00 ppm	A=(44531±643)C+(438±856) (r=0.9998)	0.04	3.33	9
IAA	Up to 500 ppb	A=(13505±4609)C+(80±18214) (r=0.9999)	2.65	0.32	6

* Abbreviations: LR: Linear range; A: Peak area; C: concentration, S_{xo}: standard deviation of the method; CV: coefficient of variation, %; N: number of calibration points.

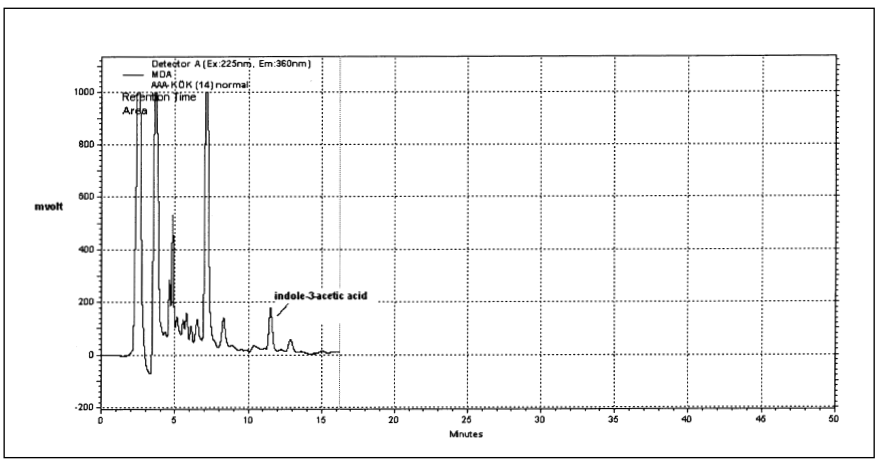


Fig. 1. Determination of indole-3-acetic acid. Column: Luna C18 (250 x 4.60 mm; 5 µ); flow rate: 0.8 ml min^{-1}. Detection: Fluorescence, (λex: 225 nm; λem: 360 nm). Mobile phase: 30% ACN, (v/v); pH: 3.0.

Hormonal Changes During Rooting of Cuttings

The hormone levels were different in respect to rootstocks during the rooting. In dormant stage, IAA level in cuttings of 5 BB was higher than in cuttings of 41 B and 420 A. In 5 BB extracts, in dormant stage, IAA amount was about 2.7 and 2.9 times higher than in 41 B and 420 A, respectively. It was determined that IAA level remained quite constant between dormant and swollen bud stages, then increased rapidly in other stages of all rootstock cuttings during rooting process (Fig. 3).

ABA was higher in 41 B and 420 A than in 5 BB in dormant stage. In 41 B and 420 A, ABA decreased rapidly until root emergency stage, then increased slightly, but this increase was not significant. However, in 5 BB, ABA remained quite constant in all stages. In root emergency and rooted stages, differences between rootstocks in respect of ABA level were not significant (Fig. 4).

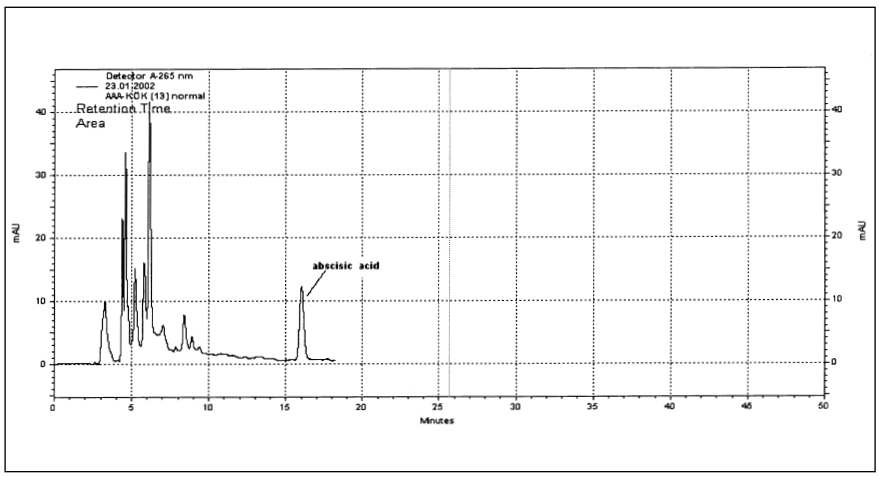


Fig. 2. Determination of abscisic acid. Column: Luna C18 (250 x 4.60 mm; 5 µ); flow rate: 0.8 ml min^{-1}. Detection: DAD, λ:265 nm. Mobile phase: 30 % ACN, (v/v); pH: 3.0.

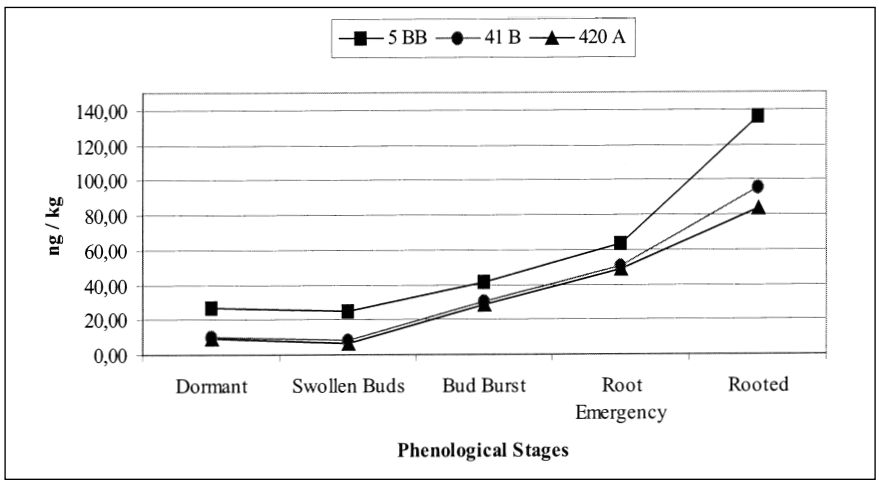


Fig. 3. Changes of endogenous IAA during rooting of cuttings.

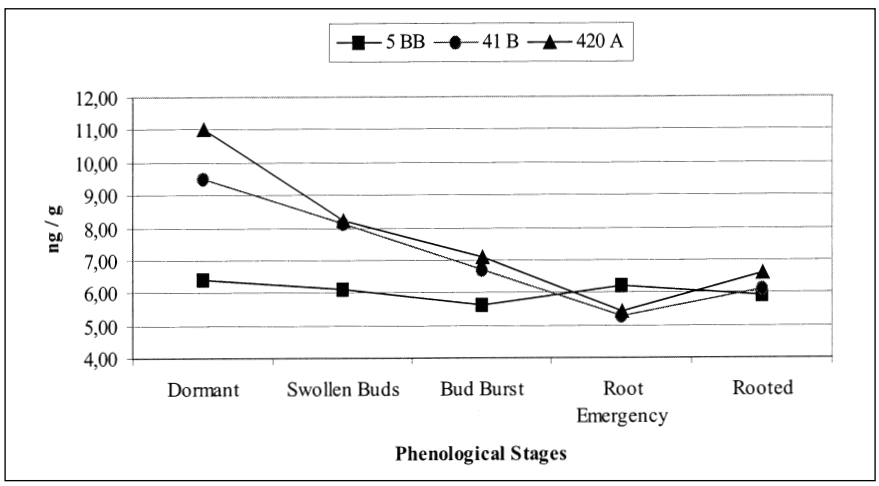


Fig. 4. Changes of endogenous ABA during rooting of cuttings.

Table 5. Correlation coefficient (r) between rooting rates and hormonal changes.

Rooting Rates	Hormonal Changes				
	Dormant	Swollen bud	Bud Burst	R. Emergency	Rooted
(For IAA)	0.92±0.15 ***	0.98±0.08 ***	0.99±0.05 ***	0.87±0.18 **	0.90±0.17 ***
(For ABA)	–0.87±0.19 **	–0.75±0.25 *	–0.84±0.20 **	0.36±0.35 NS	–0.08±0.38 NS

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Relationships Between Rooting Rates and Hormonal Levels

It was found that relationships between hormone levels and rooting rates were significant (Table 5).

IAA level has affected rooting rate positively, but ABA has affected negatively. In all stages, IAA affected rooting rate positively. On the other hand, ABA affected rooting rate negatively in dormant, swollen bud, and bud burst stages. However, the effect of ABA on rooting rates was not significant in root emergency and rooted stages.

Discussion

In the rooting process, which starts with a healing process, the formation of new cells, the induction of root formation, the linking up or several exogenous and endogenous factors will influence the success of this process. Plant hormones are of paramount importance in the multiplication process. Pending the balance of these hormones in the motherplant and in the cuttings, the rooting process will be affected either positively or adversely, but the mechanism of this physiological response has not been elucidated exactly in grapevine cuttings. Moreover, rooting of cuttings can be stimulated by some exogenously applied plant hormones (KRACKE and CRISTOFERI 1983; KAWAI 1997; KELEN and DEMIRTAS 2001).

The results suggested that different levels of IAA and ABA substances correspond to the different rooting ability of 5 BB, 41 B and 420 A hardwood cuttings. It was determined that IAA levels of rootstocks showed important differences. 5 BB cuttings, having high rooting rate, have more endogenous IAA level than 420 A and 41 B, having low rooting rate in dormant stage. Similar conclusions were drawn in the study of KRACKE et al. (1981) which indicated that different levels of endogenous hormones correspond to different rooting rate of cuttings. In addition, the lowest IAA levels were found in dormant and swollen bud stages. This result is consistent with studies of BLAKESLEY (1994) and GASPAR et al. (1994) who suggested that the initiating phase of rooting, i.e., the phase of cell division and cell differentiation, is characterized by lowering of the free auxin concentration to a minimum, whereas the expressive phase of rooting, i.e., growth and emergence of root primordia, is associated with an increase in free auxin concentration. After swollen bud stage, IAA level of all rootstocks cuttings increased during the rooting stages (Table 5). Our data confirmed the results published by BARTOLONI et al. (1986), ALVAREZ et al. (1989), KAWAI (1996) and LIU et al. (1998) who reported that endogenous IAA level increased during the rooting of cuttings. This substance is generally reported to be very active during rooting. IAA enhances the synthesis of enzymes (KRACKE et al. 1981), which induce the hydrolysis of starch and other nutrients (KRACKE et al. 1980), so they seem to be involved directly with initiation of roots and formation of vascular tissue (FUJII and NAKANO 1974; FABBRI et al. 1985). Investigations of nutrient changes during the rooting of cuttings of some grapevine rootstocks have shown that the different rooting abilities of these rootstocks correspond to different levels of protein nitrogen and to a different utilization of carbohydrate reserve (KRACKE et al. 1980; BARTOLONI et al. 1996). Difficult-to-root cuttings, proven to be very rich in starch, could be unable to utilize this reserve for rooting because of their natural low IAA activity (NANDA and ANAND 1970).

It is known that ABA generally has inhibitory effects. ABA inhibits rooting of cuttings (KRACKE et al. 1981; KAWAI 1997). In the dormant stage, it was found that cuttings of 41 B and 420 A, having low rooting rate have more endogenous ABA than cuttings of 5 BB, having high rooting rate. Our data also confirmed the results published by KRACKE et al. (1981) and CELIK (1988) which indicated that difficult-to-root rootstock cuttings have higher ABA than easy-to-root rootstock cuttings.

Conclusion

The proposed method appears to be suitable for the easy determination of these plant hormones in *Vitis* spp. The optimisation of the chromatographic conditions leads to a chromatogram in which two plant hormones are well resolved. We expect that this method can be used for analysis of these hormones in other plant materials. In this investigation, low IAA and high ABA were detected in cuttings of 41 B and 420 A, having low rooting rate, whereas high IAA and low ABA were detected in cuttings of 5 BB, having high rooting rate in dormant stage. In general, endogenous IAA increased, while ABA decreased during the rooting process. It is found that IAA has positive effect, but ABA has negative effect on rooting of cuttings.

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