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Effect of a Peat Humic Acid on Morphogenesis in Leaf Explants of *Pyrus communis* and *Cydonia oblonga*. Metabolomic Analysis at an Early Stage of Regeneration

Grazia Marino,*[,†] Valeria Righi,[‡] Andrea Simoni,[†] Luisa Schenetti,[§] Adele Mucci,[⊗] Vitaliano Tugnoli,[#] Enrico Muzzi,[†] and Ornella Francioso[†]

[†]Dipartimento di Scienze Agrarie, Università di Bologna, V.le Fanin 44, 40127 Bologna, Italy

[‡]Dipartimento di Scienze per la Qualità della Vita, Università di Bologna, Via C.so D'Augusto 237, 47921 Rimini, Italy

[§]Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, via G. Campi 287, 41125 Modena, Italy

[⊗]Dipartimento di Scienze Chimiche e Geologiche, Università di Modena e Reggio Emilia, via G. Campi 183, 41125 Modena, Italy

[#]Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy

Supporting Information

ABSTRACT: Plant regeneration is a critical step in most in vitro breeding techniques. This paper studies the effects of a low-molecular-weight humic acid (HA) on morphogenesis from pear and quince leaf explants. Variable HA amounts [0 (control), 1, 5, 10, and 20 mg C L^{-1}] were added to the regeneration media. A dose−response effect was observed in pear for root and shoot production; it was improved at HA 1 mg C L^{-1} and considerably reduced at the highest amounts. HA was, instead, ineffective in quince. The ^{1}H HR-MAS NMR analyses of calli in the induction phase showed more evident metabolite (asparagine, alanine, and γ-aminobutyric acid) signals in quince than in pear. The assignment of overlapped signals in both genotypes was supported by the 2D NMR analyses. Spectroscopic characterization suggested also an enhancement of asparagine contents in morphogenic calli of pear with respect to the control and higher HA amount treatments.

KEYWORDS: *^{1}H HR-MAS NMR analysis, 2D NMR, leaf explants, pear, quince, regeneration*

INTRODUCTION

Pear (*Pyrus communis* L.) is an important fruit crop in Europe and worldwide. Quince (*Cydonia oblonga* Mill.) is commonly used as a dwarfing rootstock for pear in Europe. However, it is characterized by graft incompatibility for some important pear cultivars and susceptibility to lime-induced iron chlorosis. The induction of somaclonal variation[1] and in vitro selection of variants with useful traits have been exploited for plant improvement in these species as an alternative tool to traditional, long-lasting breeding techniques.[2−5]

Plant regeneration from somatic tissues is a critical step in most in vitro breeding techniques; thus, much research has been aimed at determining the main factors that can improve morphogenesis.

Organogenesis is critically affected by medium composition and physical factors, although it also strongly depends on the morphogenic potential or *competence* of plant tissue cultures, which may vary with genotype, tissue age, explant choice, and polarity. In indirect organogenesis, it is unclear whether meristemoids that are formed within callus are always already determined to give origin to one specific organ or whether meristemoid and organ formation are separately and sequentially decided as a consequence of culture conditions.[6,7] Previous research showed that different interacting factors can affect shoot regeneration from leaf explants in quince[8−10] and pear,[11,12] although the latter generally displayed a low regeneration capacity.[4,13] Therefore, it seems of great significance to explore alternatives to traditional treatments to improve plant regeneration from somatic tissues.

Recent research on biological molecules acting as biostimulants has suggested new ways to promote in vivo plant growth. Biostimulants can be defined as "materials that promote plant growth when they are applied in small quantities".[14] Among these bioactive molecules, humic substances (HS) are the most investigated.

Humic substances can produce various morphological, physiological, and biochemical effects on higher plants[15−17] through the interaction with physiological and metabolic processes.[16−20] The addition of HS can stimulate nutrient uptake[21] and cell permeability[22] and seems to regulate mechanisms involved in plant growth stimulation.[18,23] Among metabolic pathways, N and S metabolism and the Krebs cycle seem to be especially affected by HS treatments.[24−26] The biological activity of the HS fraction depends on chemical structure and molecular weight. The lowest molecular weight fractions are characterized by the greatest biological activity on in vivo[16,17,21,27,28] and in vitro grown plants. In particular, a low-molecular-weight (LMW) humic acid (HA) increased root weight and respiration of kiwifruit shoot cultures;[29] it also improved rooting and reduced oxidative stress in pear.[30]

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Table 1. Basal Composition of Culture Media during Shoot Proliferation and Regeneration Phases

	shoot proliferation		regeneration			
medium	QPM[a]	PPM[a]	QIM[a]	PIM[a]	QDM[a]	PDM[a]
mineral salts						
microelements	MS[b]	QL[c]	MS	MS half-strength	MS	MS half-strength
macroelements	MS	modified QL[d]	MS	modified QL[e]	MS	modified QL[e]
glycine (μM)	26.6	53.3	26.6	53.3	26.6	53.3
growth regulators (μM)						
BA	4.4	4.4				
IBA		0.5				
TDZ			4.5	4.5	4.5	4.5
NAA			5.4	5.4		
gelling agent (g L^{-1})						
agar[f]	6.5	6.5			6.5	6.5
Phytagel			2.5	2.5		

[a]All media were enriched also with 555 μM *myo*-inositol, 2.96 μM thiamin hydrochloride, 8.1 μM nicotinic acid, 4.9 μM pyridoxine hydrochloride, and 30 g L^{-1} commercial sucrose. All compounds (but sucrose and agar) were from Sigma (Sigma-Aldrich Ltd., Milan, Italy). The pH was adjusted to 5.6−5.7 by the addition of 0.1−1 M KOH before autoclaving at 120 °C and 110 kPa for 20 min. [b]According to ref 37. [c]According to ref 38. [d]Modified QL by Leblay et al.[11] [e]Modified QL by Chevreau et al.[12] [f]Agar was A1010HA type A from DID (Difco Diagnostic International, Milan, Italy).

However, to our knowledge, the effect of HS on plant regeneration from leaf explants has never been investigated.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) has recently been used to investigate LMW substances present in biological systems. These compounds have been correlated to cellular states and responses under different stages of plant development.[31] Metabolite characterization has been applied to various biotechnological fields, including monitoring of biological processes[32] and identification of "marker" compounds. In particular, it was possible to discriminate between embryogenic and nonembryogenic calli of some higher plants.[33] ^{1}H NMR-based metabolomic analysis was found to be effective for genetic discrimination of *Catharanthus roseus* cultivars.[34] Moreover, the technique allowed the metabolic discrimination of callus cells of the same species according to their relative locations and was suggested as an objective criterion to previously select callus portions to be transferred to liquid culture, aiming to enhance the production of useful secondary metabolites.[35] In calli of *Vanilla planifolia*, one-dimensional (1D) and two-dimensional (2D) NMR techniques showed the importance of numerous compounds related with sugar mobilization and nitrogen metabolism at earlier stages of shoot regeneration and also allowed the identification of some secondary metabolites (i.e., phenolic compounds) showing enhanced accumulation during shoot differentiation.[36]

The economic impact of pear and quince and the findings reported above suggest that further investigation is needed to better understand cellular variations and responses to treatments leading to morphogenic events in these species. The present paper aims to (a) evaluate the effects of a LMW HA on morphogenesis in leaf explants of quince and pear and (b) identify by high-resolution magic angle spinning (HR-MAS) NMR analysis some compounds possibly related to morphogenic potential in leaf-derived callus at an early stage of the regeneration process.

MATERIALS AND METHODS

Plant Material. Donor shoot cultures of 'BA29' quince (*C. oblonga* L.) and 'Conference' pear (*P. communis* L.) were respectively maintained on previously experimented quince (QPM) and pear (PPM) proliferation media[13] through monthly subcultures. The former was based on a modified Murashige and Skoog[37] composition and the latter on a modified[11] Quoirin and Lepoivre[38] medium. Their complete composition is reported in Table 1. Jars were closed with twist-off metal screw caps and wrapped with polyvinylchloride (PVC) transparent film for food, allowing gas exchanges.

Standard growth conditions were as follows: 22 ± 2 °C and a 16 h photoperiod (30 μmol m^{-2} s^{-1} photosynthetically active radiation, supplied by Philips TLD 36 W/33 lamps).

Standard Regeneration Protocol. A two-step shoot regeneration protocol[13] was adopted, providing explants culture on quince (QIM) or pear (PIM) induction media, followed by their transfer respectively to quince (QDM) or pear (PDM) development media. All of them were enriched with 4.5 μM thidiazuron (TDZ); 5.4 μM naphthaleneacetic acid (NAA) was also added to QIM and PIM. The complete composition of the regeneration media is reported in Table 1.

Subapical fully expanded and not mature leaves were used as initial explants. Three transverse incisions were made on the leaves (lacking petiole), and they were placed (5 leaves per dish) with the abaxial side down in 9 cm diameter, vented polystyrene Petri dishes (Sarstedt S.R.L., Verona, Italy), each containing 20 mL of QIM or PIM medium. They were sealed with Parafilm (preventing gas exchanges) for pear and with PVC strips for quince,[13] following previous results on factors positively affecting regeneration. The explants were then transferred to QDM or PDM in Petri dishes that were sealed with PVC film for better shoot growth.[10] The shoot induction phase occurred in darkness, whereas the other phases were under standard growth conditions. Each phase lasted 30 days.

At the end of the regeneration process, the leaf explants bearing adventitious shoots were transplanted to shoot elongation media that had the same composition of QPM and PPM, but 0.9 μM 6-benzyladenine (BA) and the addition of IBA and gibberellic acid (GA_3), both at 0.5 μM. Shoots were grown in 250 mL jars (5 explants per jar; 40 mL of medium) under standard growth conditions.

Characterization of the HA Fraction. HA was extracted from sphagnum peat and purified and characterized as previously described.[39] The HA fraction with the lowest molecular weight (10 kDa) was used in the present study, as it showed the highest biological activity on in vitro grown kiwifruit shoots.[29]

Treatments with the LMW HA to the Leaf Explants. The HA amount was calculated on the basis of carbon (C) concentration. The HA solution (an HA amount corresponding to 10 mg of C solved in 100 mL of distilled water) was filter-sterilized (0.22 μm) and added to autoclave-sterilized (20 min at 120 °C) regeneration media at a final level of 0 (control), 1, 5, 10, and 20 mg C L^{-1}, respectively referred to as HA 0, HA 1, HA 5, HA 10, and HA 20 throughout the text.

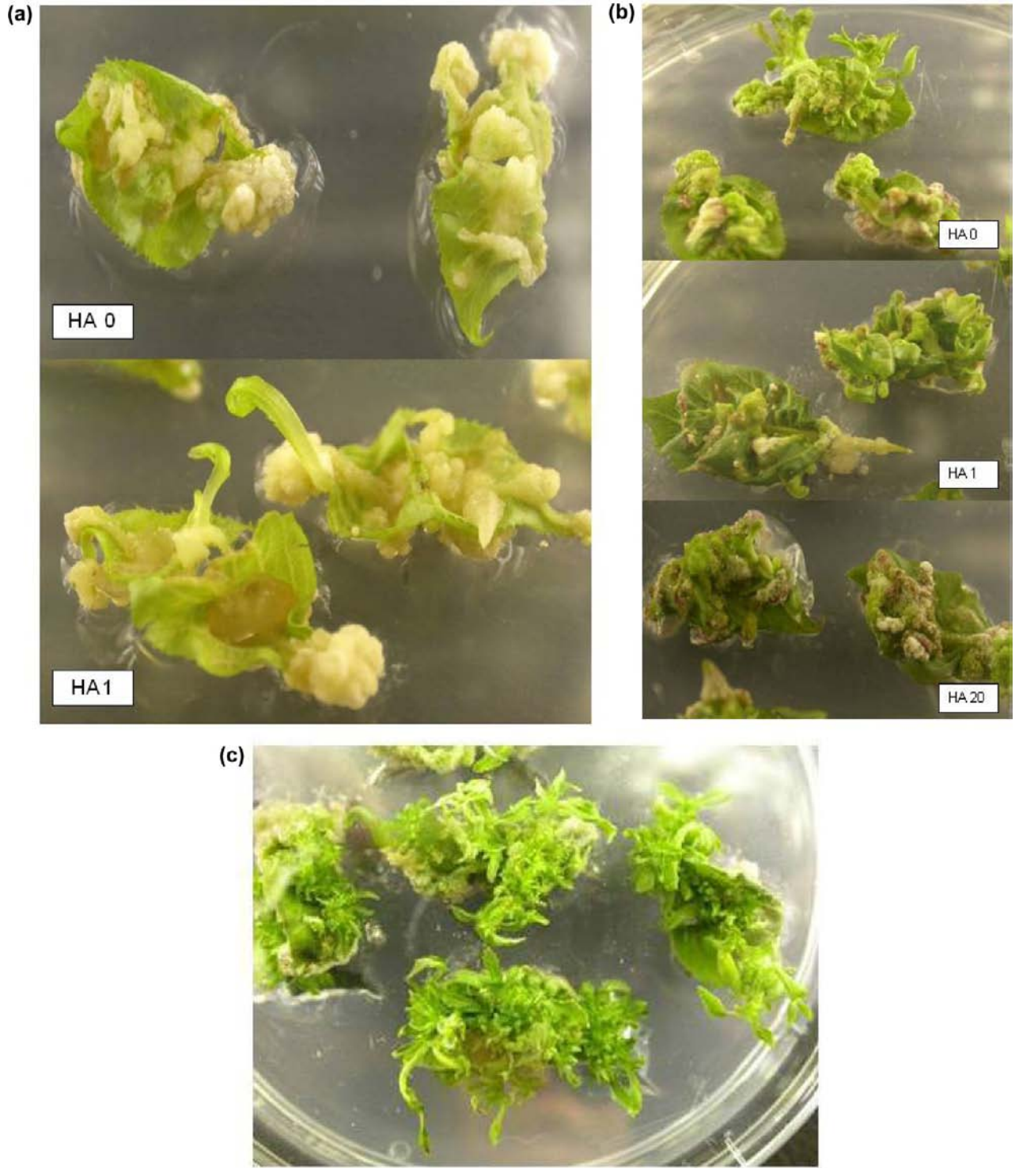

Figure 1. Effect of treatments with variable supplements of the LMW (10 kDa) HA on shoot and root regeneration at the end of (a) the induction phase and (b) the shoot development phase in leaf explants of 'Conference' pear; (c) shoot regeneration at the end of the shoot development phase in leaf explants of 'BA 29' quince.

The number of shoot-forming (SF) and root-forming (RF) leaf explants and the number of shoots (SN) and roots (RN) per cultured explant were quantified by observation under a stereomicroscope on day 30 of the induction phase and on days 15 and 30 of the shoot development phase.

The experiment was repeated a few months later with leaf explants from the same donor shoot cultures maintained on QPM (quince) and PPM (pear) standard media by monthly subcultures.

HR-MAS NMR Analysis of Leaf-Formed Callus. Callus fragments still free of visible shoots and roots were randomly collected from HA-treated leaf explants and controls of 'BA 29' and 'Conference' at the end of the induction phase. Culture conditions at this regeneration step were found to be critical in both quince[10] and pear[13] in affecting regeneration events after transfer of leaf explants to further culture phases. Callus fragments from each treatment were pooled together to have enough material for different NMR analyses. Samples were dipped in liquid N_2 and stored at −80 °C. Before NMR analysis,

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Table 2. Effects of Treatments with Variable HA Supplements in the Different Regeneration Phases

	induction				development							
	SF explants[a] dish^{-1}	SN explant^{-1}	RF explants[a] dish^{-1}	RN explant^{-1}	SF explants dish^{-1}		SN explant^{-1}		RF explants dish^{-1}		RN explant^{-1}	
HA	30 days				15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
				'Conference' Pear								
0	0.44	0.16	1.89 b[b]	1.00 ab	1.33	2.44	0.51 ab	1.76 ab	2.33 b	2.56 ab	2.09 ab	2.04 a
1	0.44	0.13	2.89 a	1.49 a	2.11	3.11	0.83 a	2.56 a	3.56 a	3.00 a	2.36 a	2.02 a
5	0.33	0.07	1.56 b	0.53 b	1.78	2.33	0.47 ab	1.36 ab	2.22 b	2.11 ab	1.22 b	0.96 b
10	0.11	0.02	1.33 b	0.42 b	0.78	2.11	0.16 b	0.78 b	2.00 b	2.11 ab	1.00 b	0.93 b
20	0.33	0.07	1.33 b	0.53 b	1.56	2.22	0.42 ab	0.73 b	1.89 b	1.89 b	1.15 b	0.91 b
significance[c]	ns	ns	**	**	ns	ns	*	*	**	*	*	**
				'BA 29' Quince								
HA												
0	2.00	1.03	–[d]	–	4.87	–	6.81	–	–	–	–	–
1	2.33	1.32	–	–	4.75	–	6.44	–	–	–	–	–
5	2.56	1.03	–	–	4.50	–	6.48	–	–	–	–	–
10	2.11	1.15	–	–	4.62	–	5.04	–	–	–	–	–
20	1.75	0.69	–	–	4.14	–	6.31	–	–	–	–	–
significance	ns	ns			ns		ns					

[a]Five leaf explants were cultured in each Petri dish. [b]For each dependent variable, means followed by different lower case letters are statistically different at $P \leq 0.05$ (Duncan's test). [c]Significance: * and **, respectively significant at $P \leq 0.05$ and $P \leq 0.01$; ns, not significant. [d]Not determined, see Results and Discussion.

a drop of D_2O was added to each sample to have deuterium for the lock system. The sample was introduced in a MAS zirconia rotor (4 mm o.d.), fitted with a 50 μL cylindrical insert to increase sample homogeneity, and then transferred into the probe cooled to 4 °C. ^{1}H and ^{13}C HR-MAS spectra were recorded with a Bruker Avance400 spectrometer operating at frequencies of 400.13 and 100.61 MHz, respectively. Experiments were performed at a temperature of 4 °C controlled by a Bruker cooling unit. Samples were spun at 4000 Hz, and three different types of 1D proton spectra were acquired by using the sequences implemented in the Bruker software: (i) a composite pulse sequence (zgcppr)[40] with a 1.5 s water presaturation during the relaxation delay, 8 kHz spectral width, 32k data points, and 32 scans; (ii) a water-suppressed spin−echo Carr−Purcell−Meiboom−Gill (CPMG) sequence (cpmgpr)[41] with a 1.5 s water presaturation during the relaxation delay, 1 ms echo time (τ), 360 ms total spin−spin relaxation delay ($2n\tau$), 8 kHz spectral width, 32k data points, and 256 scans; and (iii) a sequence for diffusion measurements based on stimulated echo and bipolar-gradient pulses (ledbpgp2s1d)[42] with Δ 200 ms, eddy current delay Te 5 ms, δ 2 × 2 ms, sine-shaped gradient with 32 G/cm followed by a 200 μs delay for gradient recovery, 8 kHz spectral width, 8k data points, and 256 scans.

2D ^{1}H−^{1}H correlation spectroscopy (COSY)[43] spectra were acquired within the 0−10 ppm region using a standard pulse sequence (cosygpprqf) and 0.5 s of water presaturation during relaxation delay, 4 kHz spectral width, 4k data points, 32 scans per increment, and 128 increments. Total ^{1}H−^{1}H correlation spectroscopy (TOCSY)[44,45] spectra were acquired within the 0−10 ppm region using a standard pulse sequence (mlevgpph19) and 100 ms of mixing time, 0.5 s of water presaturation during relaxation delay, 4 kHz spectral width, 4k data points, 128 scans per increment, and 128 increments. 2D ^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC)[46] spectra were acquired using a standard echo−antiecho pulse sequence (hsqcedetgp) and 0.5 s of relaxation delay, 1.725 ms of evolution time, 4 kHz spectral width in f2, 4k data points, 128 scans per increment, 15 kHz spectral width in f1, and 256 increments.

NMR spectra were processed using Xwinnmr software (Bruker). A line-broadening apodization function of 1 Hz was applied to ^{1}H HR-MAS data prior to Fourier transformation in the case of 1D spectra. A sine apodization function in both dimensions was employed for COSY spectra. A cosine squared apodization function in both dimensions was employed for TOCSY spectra. A line-broadening apodization function of 10 Hz in f2 and cosine squared apodization function in f1 were employed for HSQC spectra. NMR chemical shifts were referenced with respect to β-glucose (Glc): 4.64 and 96.6 ppm for ^{1}H and ^{13}C, respectively.

Integral values were obtained from water-presaturated ^{1}H NMR spectra (zgcppr) after baseline correction and normalization of the total spectral area between 9 and 0 ppm. Accuracy of ±5% can be assumed.

Statistical Analysis. The data on regeneration were subjected to an analysis of variance in a randomized factorial design. For each genotype and regeneration phase, they are based on four and five Petri dishes (replicates) per treatment, respectively, in the first and second experiments. Values for RF- and SF-explants number per dish and shoot and root number per explant are means of the two experiments; before being processed for statistical analysis, they were transformed to $y = \sqrt{(x + 0.5)}$, where x is the original value, for variance normal distribution.[47] Mean values were separated by Duncan's test at $P \leq 0.05$. Normalized integral values of NMR peaks of the more evident signals were performed for principal components analysis (PCA) of HA levels and genotypes.[48]

RESULTS AND DISCUSSION

Characterization of the HA Fraction. The HA fraction was previously characterized using NMR analysis.[39] In brief, the sample was dominated by the following ^{13}C chemical shifts: 190−160 ppm (carbonyls of ketones, quinines, aldehydes, and carboxyls); 160−140 ppm (phenols and aromatic C-substituted); 140−110 ppm (unsubstituted aromatic C and olefinic C); 110−95 ppm (anomeric C); 65−45 ppm (*O*-alkyl C ; *N*-alkyl C); and 45−0 ppm (alkyl C).

This fraction differed from the HMW fraction for higher signals in phenol and carboxyl C. In addition, the carboxylic concentration determined by potentiometric titration ($COOH_{LMW} \approx 5.0$ mequiv g^{-1} vs $COOH_{HMW} \approx 4.0$ mequiv g^{-1}) supported the NMR result.[29]

Morphogenesis and Shoot Development. Shoot and root regeneration occurred via callus formation in the explants of both genotypes.

'Conference' Pear. Most regenerating explants produced shoots and roots at the same time; sporadic leaves also appeared on the explants without apparent shoot formation (Figure 1a). However, in the induction phase, the most evident effect of HA was an earlier root formation from callus, whereas shoot regeneration was very low and not affected by treatments (Table 2). In particular, the number of RF-explants was significantly enhanced at HA 1 with respect to the control. This is mostly consistent with the high content of phenolic compounds in the HA fraction, and the well-known general positive effect of HS and, especially, of polyphenolic substances on rooting.[49,50] However, the average RN per explant at HA 1 was not statistically different from the control, probably due to the high variability of this trait within replicates at the low HA level. Instead, it decreased at HA 5 with respect to HA 1 and thereafter remained statistically comparable to the highest HA levels.

On day 15 of the development phase, the number of RF-explants was still highest with HA 1; the RN generally almost doubled with respect to the previous culture phase, being again higher with HA 1 than other HA amounts, although not different from the control. The SN also increased especially with HA 1, which was significantly different from HA 10 (Table 2).

Due to previous trials showing a longer time course of the shoot regeneration process in pear than in quince,[13] the development phase was extended to day 30 of culture. At that time the SN per explant with HA 1 was higher than HA 10 and HA 20 and almost 50% higher than the control, although not significantly (Table 2). Since some roots degenerated or were included in the growing callus, the number of RF-explants and the RN were in many cases lower than on day 15. However, at HA 1 they were still respectively higher than HA 20, and HA 5 to HA 20, although comparable to the controls (Table 2). Moreover, the occurrence of some explant browning at the highest HA levels was proof of a certain tissue stress (Figure 1b). These results partially agree with previous data on the positive effect of the same HA at low doses (0.5−1) on root development in in vitro kiwifruit shoots and, in contrast, the adverse effect of higher HA amounts especially on shoot growth.[29]

'BA 29' Quince. Ten-fold more shoots than in pear (and just sporadic roots) were present on leaves at the end of induction (Table 2).

On day 15 in the development phase, the SN per explant was in a range of about 5−7 compared to maxima of 0.8 and 2.6, respectively, on days 15 and 30 in pear. However, differing from pear, HA did not have significant effects in either phase.

Data on day 30 of this phase are not reported for quince as, due to the very high number of regenerated shoots, it was difficult to distinguish those derived from single original adventitious buds from those that developed later through axillary bud proliferation (Figure 1c).

After leaf explant transfer to the shoot elongation media, a positive carry-over effect of HA 1 on the number of explants with transplantable (>5 mm) shoots per jar was found with respect to HA 20 (3.1 and 1.1 explants jar^{-1}, respectively; significant at $P \leq 0.05$) in pear. Instead, no effect was found in quince (data not reported in tables). Well-developed shoots of both genotypes could be successfully transplanted to further proliferation cycles and rooted to give origin to somaclones.

HR-MAS NMR Analysis. HR-MAS NMR permits the study of intact tissues providing their detailed metabolic composition. It enables any pretreatment on the sample, such as extraction and separation, to be avoided and the signals from various components to be detected simultaneously.[51] In this study, HR-MAS NMR spectroscopic analysis was performed on calli at a semiquantitative level, to highlight the major metabolic changes induced by HA treatment.

The ^{1}H HR-MAS NMR spectra of callus samples from leaf explants grown on control media and after treatment with HA are shown in Figures 2 and 3. Some metabolites can be readily

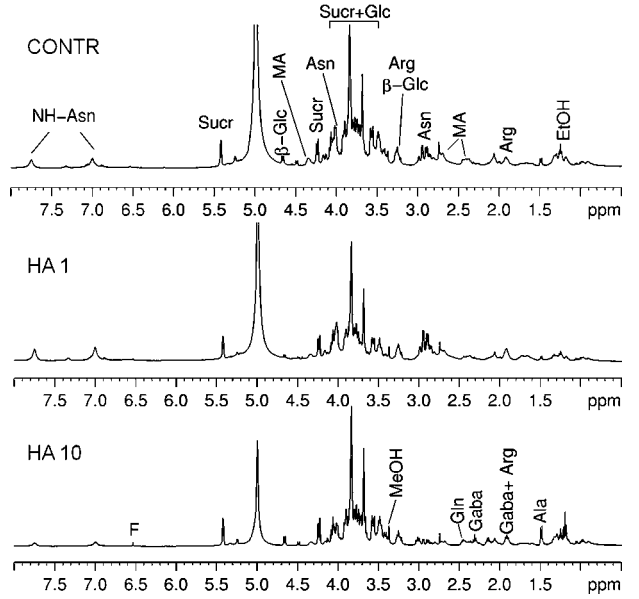

Figure 2. HR-MAS ^{1}H NMR spectra of leaf-derived callus samples of controls (CONTR, without addition of HA) and explants treated with HA 1 and HA 10 of 'Conference' pear at day 30 in the induction phase.

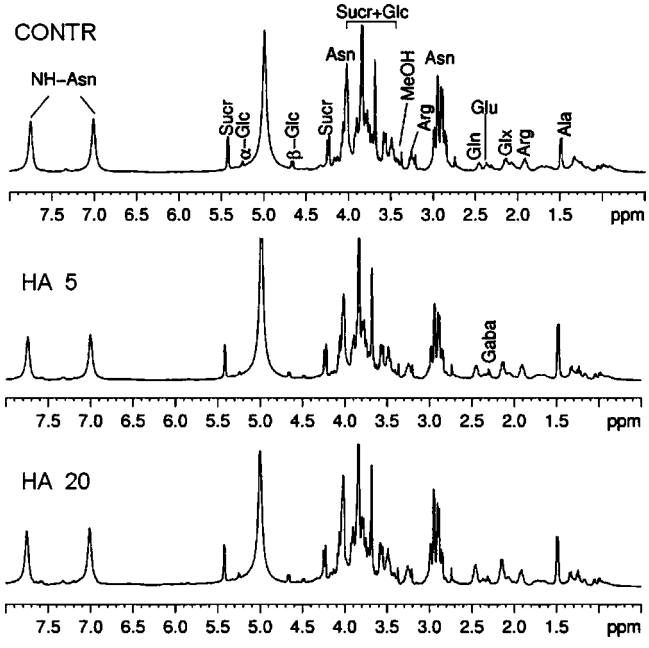

Figure 3. HR-MAS ^{1}H NMR spectra of leaf-derived callus samples of controls (CONTR, without addition of HA) and explants treated with HA 5 and HA 20 of 'BA 29' quince at day 30 in the induction phase.

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assigned by direct inspection of 1D ^{1}H NMR spectra, whereas 2D NMR analysis was performed to identify metabolites having signals that overlapped in the 1D ^{1}H NMR spectra for both pear and quince. COSY spectra are reported in Figures 4 and 5, whereas TOCSY and HSQC spectra are reported in the Supporting Information (Figures S1 and S2).

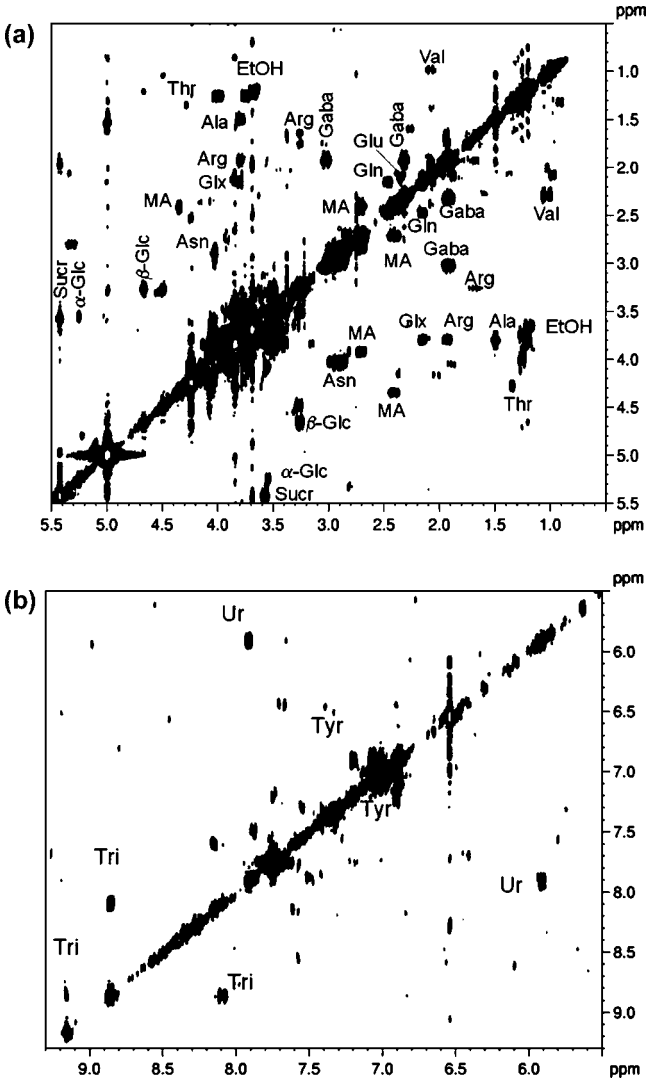

Figure 4. 2D ^{1}H−^{1}H COSY spectrum of 'Conference' pear control: (a) aliphatic region; (b) aromatic region.

A close inspection of COSY (Figures 4 and 5), TOCSY (Supporting Information, Figure S1), and HSQC (Supporting Information, Figure S2) spectra allowed us to identify the complete metabolic pattern of calli. Main signals due to sugar molecules [sucrose (Sucr), Glc, and fructose (Fru)] are detected in the region from 5.42 to 3.25 ppm, but not discussed because of the high Sucr content in the media used for maintenance of the donor plants and for culture of the leaf explants in all of the regeneration phases. Moreover, it is well-known that a partial hydrolysis of Sucr occurs during autoclave sterilization of culture media, and further Sucr inversion to Glc and Fru takes place during plant in vitro culture.[6]

The analysis of homonuclear 2D spectra made it possible to assign signals from alanine (Ala), arginine (Arg), asparagine (Asn), ethanol (EtOH), glutamine (Gln), glutamate (Glu), γ-aminobutyric acid (Gaba), lysine (Lys), valine (Val), and malic acid (MA) and low signals from trigonelline (Tri), tyrosine (Tyr), threonine (Thr), histidine (His), and uridine (Ur). The ^{1}H−^{13}C HSQC NMR spectra, besides confirming the presence of the above metabolites, allowed the identification of choline-containing compounds (ChoCC), methanol (MeOH), and *myo*-inositol (Myo) on the basis of both proton and carbon chemical shifts. It is worth emphasizing that the two broad resonances at 7.0 and 7.8 ppm probably do not derive from polyphenols[52] in our samples, but from NH of Asn, as suggested by the correct integral ratio with $CH_2\beta$ at 2.9 ppm (Supporting Information, Figure S3), by the lack of H−C correlation in HSQC spectra of quince (Supporting Information, Figure S4), where the signals are much higher than in pear, and by the presence of a NH−NH correlation and a low correlation between NHs and $CH_2\beta$ at 2.9 ppm in TOCSY spectra of quince (Supporting Information, Figure S5). The detection of these high signals from NH protons, in the presence of added D_2O, indicates that proton−deuterium exchange does not take place at an appreciable level during the experiment. Hence, these signals probably derive from Asn in the tissue. Indeed, they were also observed in 1D diffusion-

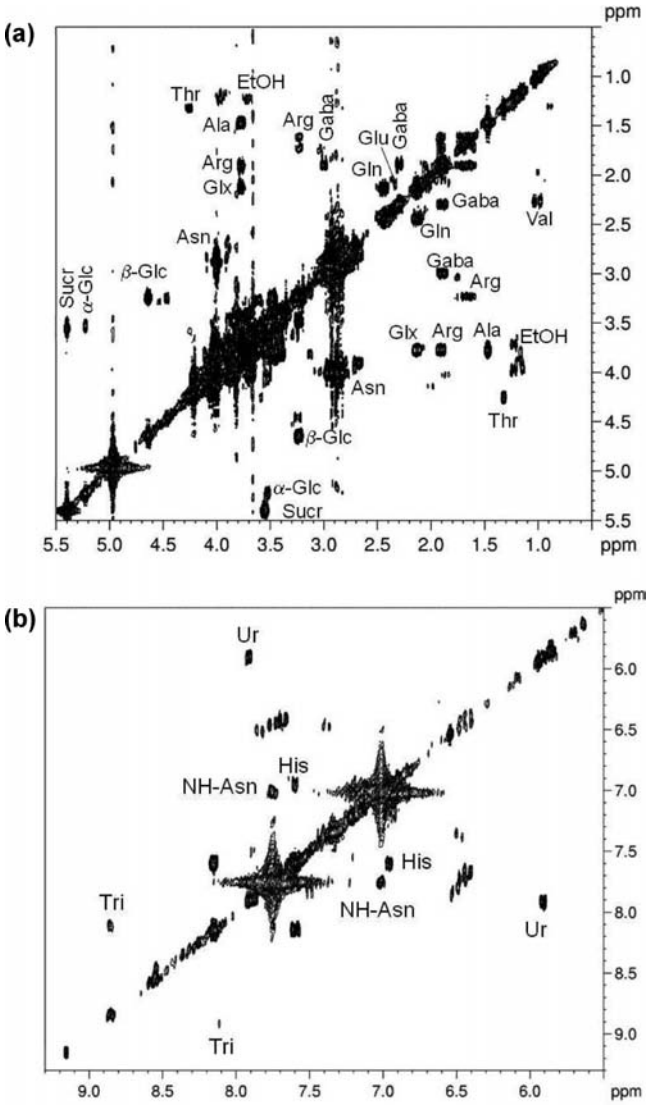

Figure 5. 2D ^{1}H−^{1}H COSY spectrum of 'BA 29' quince control: (a) aliphatic region; (b) aromatic region.

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edited spectra (Supporting Information, Figure S6), where resonances from slowly diffusing molecules are retained at the expense of those from rapidly diffusing species, which are in turn reduced (usually LMW metabolites). Signals of LMW molecules are found in 1D diffusion-edited spectra only when these molecules undergo restricted motion, due to the subcellular environment.[53] The detection of the two signals at 7.8 and 7.0 ppm (Supporting Information, Figure S6), together with CH_2 around 2.9 ppm, seems to indicate that Asn is subjected to restricted motions due to compartmentation, at least to a certain extent.

The overall NMR data show that the ^{1}H HR-MAS spectra of pear calli are characterized by signals from Ala, Arg, Gaba, Asn, MA, MeOH, EtOH, and fumarate (F, traces). Some changes with respect to the control are observed after treatment with HA (Figure 2). The sample grown with HA 1 shows a ^{1}H NMR profile very similar to that of the control, with an increase of Asn signals. An enhancement of F, Gaba, Ala, and EtOH, a slight decrease of MA, and a strong decrease of Asn signals are displayed with HA 10 (Supporting Information, Figure S7). These features are confirmed by CPMG spectra (Supporting Information, Figure S8) that filter out broad rapidly relaxing resonances, improve the resolution, and make it possible to better detect signals from slowly relaxing and rapidly tumbling species.

The metabolic profile of quince calli obtained by ^{1}H HR-MAS (Figure 3) is always dominated by Asn signals, whereas Ala, Gln, and Glu are more evident than in pear spectra. However, we could not detect relevant changes in the metabolic profile after treatment with different HA concentrations, except a slight increase in Gln signals (Supporting Information, Figure S9), as confirmed by the CPMG spectra (Supporting Information, Figure S10). In quince samples low resonances from a benzoate ester were also found in the aromatic region.

PCA scatterplot (Figure 6) shows differentiation between HA concentrations and genotypes. In particular, Asn and NH-Asn are negatively correlated to EtOH in both genotypes. However, quince shows the highest Asn and NH-Asn levels and the lowest EtOH concentrations; in contrast, pear is characterized by the lowest Asn and NH-Asn levels and the highest EtOH concentrations. Moreover, a close relationship between pear Asn and NH-Asn signals and the HA 1 dose is shown.

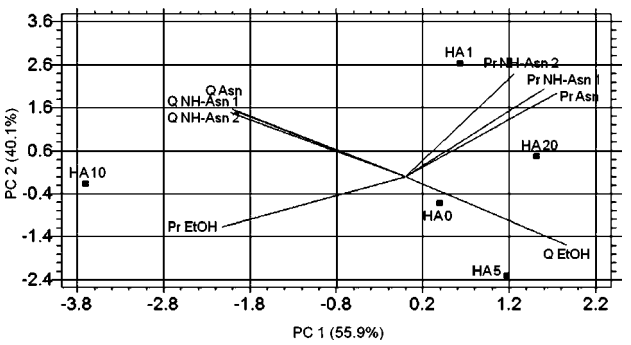

Figure 6. PCA scatterplot of the ^{1}H NMR spectra of Asn, NH-Asn 1 (7.0 ppm), NH-Asn 2 (7.8 ppm), and EtOH of quince 'BA 29' (Q) and pear 'Conference' (Pr), treated with different HA supplements, against the two principal components PC 1 and PC 2.

Asn has sometimes been included in culture media, and some studies showed its effectiveness in stimulating morphogenesis.[6,54] It was also related to in vivo plant growth; in germinating seeds of legumes, Asn was found as a main storage protein of the developing axis.[55] Moreover, Asn can be a precursor of ethylene, as in plants it contributes to produce methionine.[56] With regard to this action, it should be noted that in our previous research[13] ethylene inhibition in the induction phase strongly reduced organogenesis in pear leaf explants. These findings are partially in agreement with our previous and present results; that is, higher Asn signals at HA 1 than in other treatments in pear calli should be associated with the highest RF and RN numbers under the present experimental conditions. However, these differences in regeneration rates were only significant with respect to the highest HA levels and not the control, but RF-explants in the induction phase.

An increase of EtOH signal at high HA levels was instead related to lower regeneration in pear. This is consistent with results reported on in vitro cultured shoots of *Prunus avium*, where EtOH production was associated with stress and senescence symptoms.[57]

Mixtures of aliphatic amino acids such as Ala, Gln, and Asn were considered to be biologically active and effective in promoting embryogenesis in callus and cell suspensions of *Daucus carota*; however, these amino acids may be ineffective or inhibitory in the morphogenesis of another species.[6] The higher levels of Ala, Gln, and Asn in quince than in pear are related to the higher shoot regeneration rates of the former with respect to the latter.

In conclusion, the present research confirms high and low regeneration rates, respectively, for quince and pear. In particular, a low genotype-specific regeneration capacity for pear was previously assumed, based on data on patterns of ethylene and polyamine production and responses to aminoethoxyvinylglycine,[13] and led to the present evaluation of the comparative effects of HA on morphogenesis of these two genotypes. HA effects on morphogenesis in 'Conference' pear partially agree with previous data on improved growth of in vitro kiwifruit[29] and pear plantlets and reduced oxidative stress in pear shoots[30] after treatments with the same HA (0.5−1) used in this study. Instead, HA was ineffective in quince. In addition, these results suggest that in our culture conditions Asn might be associated with enhanced morphogenic capacity of callus in both plant genotypes. This is consistent with data reported for calli of *V. planifolia*, where some secondary metabolites, including Asn, accumulated to a greater extent during shoot differentiation.[36]

Plant regeneration protocols mostly provide subsequent subcultures of explants from induction to shoot development and elongation media. Culture conditions in the induction phase were found to be critical both in quince[10] and in pear[13] in affecting regeneration after leaf explant transfer to further culture phases. It should be pointed out that for HR-MAS NMR very small tissue samples are required. Therefore, this technique might be useful in selecting optimal treatments at the early induction stage and the leaf explants to be transferred to development and elongation media, thus saving chemicals and labor and improving plant regeneration efficiency also in quince and in pear.

■ ASSOCIATED CONTENT

Supporting Information

Additional TOCSY, HSQC, ^{1}H diffusion-edited, CPMG NMR spectra of calli and integrals of selected ^{1}H NMR signals. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39-51-2096428. Fax: +39-51-2096400. E-mail: grazia.marino@unibo.it.

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Notes

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■ ABBREVIATIONS USED

Ala, alanine; Arg, arginine; Asn, asparagine; BA, 6-benzyladenine; CPMG, Carr−Purcell−Meiboom−Gill; ChoCC, choline-containing compounds; COSY, correlation spectroscopy; EtOH, ethanol; 1D, one-dimensional; 2D, two-dimensional; Fru, fructose; GA_3, gibberellic acid; Gln, glutamine; Glu, glutamate; α-Glc, α-glucose; β-Glc, β-glucose; HA, humic acid; His, histidine; IBA, indole-3-butyric acid; MA, malic acid; MeOH, methanol; HR-MAS, high-resolution magic angle spinning; HSQC, heteronuclear single-quantum coherence; HS, humic substances; LMW, low molecular weight; Lys, lysine; NAA, α-naphthaleneacetic acid; NMR, nuclear magnetic resonance; Sucr, sucrose; PAR, photosynthetically active radiation; PDM, pear development medium; PIM, pear induction medium; PPM, pear proliferation medium; PVC, polyvinylchloride; QDM, quince development medium; QIM, quince induction medium; QPM, quince proliferation medium; RF, root forming; RN, root number; SF, shoot forming; SN, shoot number; Sucr, sucrose; TDZ, thidiazuron; Thr, threonine; TOCSY, total correlation spectroscopy; Tri, trigonelline; Tyr, tyrosine; UDP, uridine-monophosphate; Ur, uridine; Val, valine

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