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ARTICLE

Determination and Confirmation of Nicotinic Acid and Its Analogues and Derivates in Pear and Apple Blossoms Using High-Performance Liquid Chromatography−Diode Array−Electrospray Ionization Mass Spectrometry

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Erwinia amylovora causes fire blight, a serious disease of apple and pear. The bacterial pathogen colonizes the flower stigma and hypanthium, where it multiplies and then invades through natural openings (nectarthodes). *E. amylovora* requires nicotinic acid as growth factor, and competition for nicotinic acid is being explored as a novel biocontrol strategy. The ability of *E. amylovora* to substitute nicotinic acid with analogues or derivates as growth factors has not been investigated yet. Furthermore, the presence and/or variable concentration of nicotinic acid and its analogues/derivates in the hypanthium could be associated with the different susceptibilities to fire blight of hosts and nonhosts and with the differential sensitivity to the disease among apple and pear varieties. Currently, no methods to specifically quantify nicotinic acid and nicotinic acid analogues/derivates in the hypanthium of apple and pear blossoms are available. This study demonstrates that *E. amylovora* can grow using nicotinamide and 6-hydroxynicotinic acid as alternative growth factors to nicotinic acid, but not using 2-hydroxynicotinic acid. A novel HPLC/ES-MS method was developed for the detection and quantification of nicotinic acid and its analogues/derivates directly in the hypanthium of apple and pear blossoms. Analyses established the presence of nicotinic acid and nicotinamide, whereas no detectable amounts of 6-hydroxynicotinic acid and 2-hydroxynicotinic acid were observed. Mean nicotinic acid content in the pear hypanthium was found to be approximately 2 orders of magnitude higher than in the apple hypanthium, which may contribute to the differential susceptibility of these two host species to fire blight. Contents of nicotinamide were in contrast similar. Nicotinic acid can therefore be considered a relevant factor in the pathogen establishment in pear blossoms, whereas nicotinamide could cover a primary role in apple blossoms.

KEYWORDS: Nicotinic acid; nicotinamide; 6-hydroxynicotinic acid; 2-hydroxynicotinic acid; Rosaceae; *Malus*; *Pyrus*; hypanthium; *Erwinia amylovora*; fire blight

INTRODUCTION

Fire blight, caused by the enterobacterium *Erwinia amylovora*, is one of most devastating bacterial diseases affecting economically important cultivation of apple and pear and numerous amenity/native Rosaceae species worldwide (*1*, *2*). The blossom blight phase of the disease is initiated by epiphytic populations of *E. amylovora* that colonize the stigma. The subsequent movement of the bacteria to the floral cup (hypanthium) is facilitated by rain or heavy dew, where the pathogen invades the host via natural openings called nectarthodes, and then rapidly spreads endophytically to cause necrotic death of plant tissues (*3*−*5*). Apple and pear nectar is an excellent medium for bacterial multiplication (*3*) and has been shown to be a chemoattractant for *E. amylovora* (*6*). Prevention of the blossom infection is fundamental in fire blight management (*1*), and therefore the understanding of the chemical environment of the infection court could be important for the development of new fire blight control strategies.

E. amylovora requires nicotinic acid (NiAc) as growth factor in laboratory culture media (*7*). As a practical implication, degradation of NiAc and consequent inhibition of *E. amylovora* growth by microbial biocontrol agents have been recently proposed as a new approach for the control of the disease (*8*). To date, however, the presence and quantity of NiAc in apple and pear nectar have not been documented. Verifying the possibility of *E. amylovora* using alternative growth factors to NiAc, such as NiAc analogues or derivates, is also indispensable. Nicotinamide ($NiNH_2$), an analogue, is the amide form of NiAc. 6-Hydroxynicotinic acid

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(6-HNiAc) (*9*) and 2-hydroxynicotinic (2-HNiAc) (*10*, *11*) are, instead, first intermediate metabolites produced by microorganisms during NiAc degradation.

HPLC-based methods are commonly applied to quantify NiAc and $NiNH_2$ in various food substrates (*12*−*15*), including infant milk (*16*), compound feed (*17*), meat (*18*, *19*), and coffee (*20*, *21*). HPLC-based methods have also been widely used to analyze the nectar chemical composition in many plant species to catalogue the sugar composition (*22*−*25*) and its temporal changes (*26*), to assess amino acid composition (*27*, *28*), and to evaluate the role of taste and plant secondary compounds in the ecology of pollination (*29*, *30*). The chemical composition of pear and apple stigma exudates has been characterized, giving particular attention to the identification of specific free sugars, free amino acids, and polysaccharide subcomponents available as carbon and nitrogen sources for microbial activity (*31*). Currently, no methods are available to specifically quantify NiAc or $NiNH_2$, 6-HNiAc and 2-HNiAc directly in the hypanthium.

The aims of this work were the assessment of the capacity of *E. amylovora* to utilize NiAc analogues/derivates as possible alternatives to NiAc and the development of a rapid and robust HPLC-MS method for their confirmation and quantification in the hypanthium of apple and pear blossoms. To our knowledge, the present method represents the first attempt of quantification of a vitamin directly in the blossom hypanthium.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions. *E. amylovora* CFBP-1430 (*32*) was used in the growth experiments. It was cultured on diluted (10%) tryptic soy agar (TSA; Difco, Detroit, MI) at 24 °C.

Utilization of Nicotinic Acid Analogues and Derivatives as Growth Factors by *E. amylovora*. $NiNH_2$, 6-HNiAc (Fluka, Buchs, Switzerland), and 2-HNiAc (Alfa Aesar, Ward Hill, MA) were tested as possible alternative to NiAc by *E. amylovora*. NiAc is required by the pathogen in minimal amounts; therefore, to eliminate traces of NiAc from bacterial inoculum, the pathogen was precultivated overnight at 24 °C, with agitation (150 rpm), in M9 minimal medium (*33*) supplemented with 0.3 mM thiamin−hydrochloride (Fluka). Growth was monitored using the BioscreenC MBR system (Growth Curves Oy, Helsinki, Finland). Aliquots of 200 μL of the same medium amended with 0.8 mM NiAc, $NiNH_2$, 6-HNiAc, or 2-HNiAc were dispensed aseptically into honeycomb microplates (10 × 10) of Bioscreen C. M9 medium supplemented only with 0.3 mM thiamin−hydrochloride was used as control. For each treatment, five successive wells of the same column were filled with the medium, and the first four wells were inoculated with *E. amylovora* at an initial concentration of about 3×10^6 CFU/mL corresponding to an optical density at 600 nm (OD_{600}) of 0.003 ± 0.002. The fifth well was used as blank control in the measurement. Plates were incubated at 24 °C for 3 days, and *E. amylovora* growth was determined by measuring the optical density with a wideband filter (420−580 nm) at 15 min intervals. Each reading was preceded by a vibration shaking of 10 s to avoid cell sedimentation and to favor aeration.

Blossom Samples. In 2008, blossoms of different cultivars of pear (*Pyrus communis*), apple (*Malus domestica*), Japanese crab apple (*Malus floribunda*), and crab apple (*Malus sylvestris*) were collected from several orchards in the Non Valley, northern Italy. Samples of the pear cultivars Spadona and Williams were collected in two different places each and are therefore indicated with the suffixes 1 and 2 depending on the provenience (**Table 1**). Whole branches bearing closed blossoms, randomly chosen within the canopy of trees of the same orchard, were collected in the evening and brought directly to the laboratory. Opened blossoms were removed, whereas closed blossoms were allowed to open overnight in the laboratory and used the day after in the experiments.

Standard Solutions. A standard solution containing 10 mg/L of, respectively, NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc was used in the preliminary tests for the selection of the most appropriate analytical column and conditions of separation/detection. Chemical structures and optimized chromatographic separation of these compounds are given in **Figure 1**.

In the recovery trial was used a standard solution containing NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc (1 g/L of each compound) dissolved in 1% formic acid (HPLC-grade, Carlo Erba, Milano, Italy) in Millipore water (Milli-Q system, Millipore, Bedford, MA) (pH 2.1). The standard solution was stored in darkness at 4 °C. For the calibration curve, seven solutions, covering the range from 0.08 to 4 mg/L, were prepared in 1% formic acid by dilution of a stock solution containing 100 mg/L of NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc. The solutions were freshly prepared just before the injection.

Sample Preparation and Extraction. Each sample consisted of 15 blossoms randomly selected on the overnight-bloomed branches. Subject of analysis was the blossom hypanthium, constituted from the base of the sepals, petals, and stamens fused together and typical of Rosaceae. Petals, stamens, and stigmas were therefore removed from the blossoms, and the remaining part was weighed using a Mettler Toledo PL3002 balance (Greifensee, Switzerland) prior to proceeding with the extraction. In the recovery trial, blossoms of pear (cv. Luise Bonne) and apple (cv. Golden Delicious) were used. For each fortified sample, two blossoms were amended with 5 μL of the standard solution (1 g/L of NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc). Amended blossoms were left to dry in a laminar flow hood and then put together in a glass tube with the remaining blossoms constituting the same sample.

The extraction consisted of 5 min of ultrasonication followed by 10 min of extraction in 3.3 mL of 1% formic acid in water, performed three times on the same sample. The three extractions/sample were pooled, adjusted to an exact volume of 10 mL, and filtered through a 0.22 μm filter (Millex-GV, Millipore) into HPLC vials. The vials were kept at −20 °C until analysis.

HPLC-DAD-MS Conditions. Mass spectrometric analysis was carried out on a Micromass ZQ LC-MS system (Micromass, Manchester, U.K.), equipped with a Waters 2690 HPLC system, a Waters 996 DAD detector, and Empower software (Waters Corp., Milford, MA). Separation was performed using an Atlantis dC18 column (250 mm × 4.6 mm, 5 μm; Waters Corp.) coupled with an Atlantis dC18 precolumn (20 × 4.6 mm, 5 μm). Formic acid and methanol (HPLC-grade, Carlo Erba) and Millipore water (Millipore) were used for chromatography. The mobile phase consisted of 1% formic acid in water (A) and 1% formic acid in methanol (B). Separation was carried out at 30 °C in 25 min, under the following conditions: 0% B for the first 5 min, linear gradients to 37.5% B in 15 min and to 100% B in 1 min, 100% B for 4 min, 0% B in 10 s. The column was equilibrated for 8 min prior to each analysis. A flow rate of 0.9 mL/min and an injection volume of 20 μL were used. The UV−vis spectra were recorded from 210 to 600 nm, with detection at 260 nm for NiAc, $NiNH_2$, and 6-HNiAc, whereas at 320 nm for 2-HNiAc. MS conditions were set as follows: capillary voltage, 3000 V; extractor voltage, 6 V; source temperature, 105 °C; desolvation temperature, 200 °C; cone gas flow (N_2), 30 L/h; and desolvation gas flow (N_2), 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyzer. Electrospray mass spectra ranging from m/z 20 to 800 were taken in positive mode with a dwell time of 0.1 s, at CV 10, 20, and 40 V. Different cone voltages were used to obtain different fragmentation patterns of each compound and compare them to the fragmentation pattern of the respective external standards. Compounds were identified on the basis of their retention time, UV and MS spectra, and molecular ion identification. Quantification was performed with MS in SIM mode (CV 20 was used because it gave the highest signal) at m/z 123.6 for NiAc, m/z 139.7 for both 2-HNiAc and 6-HNiAc and m/z 122.6 for $NiNH_2$ using the external standard method. Under these conditions retention times were $NiNH_2$, 5.9 min; NiAc, 6.3 min; 6-HNiAc, 13.3 min; and 2-HNiAc, 14.8 min. For each compound, values were corrected according to the calculated recovery and expressed in micrograms per gram of cleaned blossoms.

Accurate mass measurements were performed on Acquity-Synapt LC-Q-TOF (Waters Corp.). LC conditions were the same as described above. The settings of Q-TOF were as follows: ESI positive mode; mass range, 50−1000 Da; double W; capillary voltage, 3 kV; sampling cone, 25 V; extraction cone, 3 V; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 40 L/h; desolvation gas flow, 1000 L/h. The compound used as reference for lock mass correction was leucine/eukephalin.

RESULTS AND DISCUSSION

Utilization of Nicotinic Acid Analogues/Derivatives as Growth Factors by *E. amylovora*. Similar growth curves were obtained

Table 1. Description of Samples Analyzed in the Study

species/variety	provenience	geographic coordinates	elevation[a] (m)	age (years)	samples[b] (no.)	mean sample weight (g ± SD[c])
Spadona 1	Rumo	46° 26′ 31″ N; 11° 01′ 48″ E	1087	~60	4	1.45 ± 0.04
Spadona 2	Smarano	46° 20′ 35″ N; 11° 06′ 18″ E	962	~60	6	2.05 ± 0.09
Williams 1	Cles	46° 21′ 46″ N; 11° 01′ 55″ E	655	~50	7	1.10 ± 0.04
Williams 2	Fondo	46° 26′ 31″ N; 11° 07′ 41″ E	915	~50	6	1.02 ± 0.02
Louise Bonne	Cles	46° 21′ 34″ N; 11° 01′ 53″ E	655	~80	10	1.13 ± 0.06
crab apple	Taio (loc. Sabino)	46° 16′ 47″ N; 11° 03′ 49″ E	380	~30	3	1.11 ± 0.05
Japanese crab apple	Smarano	46° 20′ 18″ N; 11° 06′ 22″ E	998	12	4	0.52 ± 0.03
Golden Delicious	Campodenno (loc. Crescino)	46° 14′ 46″ N; 11° 03′ 23″ E	277	11	10	0.93 ± 0.12
Stark Delicious	Taio (loc. Dermulo)	46° 20′ 01″ N; 11° 03′ 51″ E	543	~30	6	1.28 ± 0.29
Fuji	Cagnò	46° 23′ 27″ N; 11° 02′ 28″ E	635	5	4	1.27 ± 0.05
Canada Reinette	Taio (loc. Dermulo)	46° 20′ 01″ N; 11° 03′ 51″ E	543	~60	6	1.74 ± 0.08
Pinova	Cagnò	46° 24′ 13″ N; 11° 02′ 01″ E	709	8	6	0.97 ± 0.03
Braeburn	Cagnò	46° 24′ 11″ N; 11° 02′ 02″ E	709	5	3	1.12 ± 0.06

[a] Above sea level. [b] Each sample consisted of 15 blossoms. [c] Standard deviation.

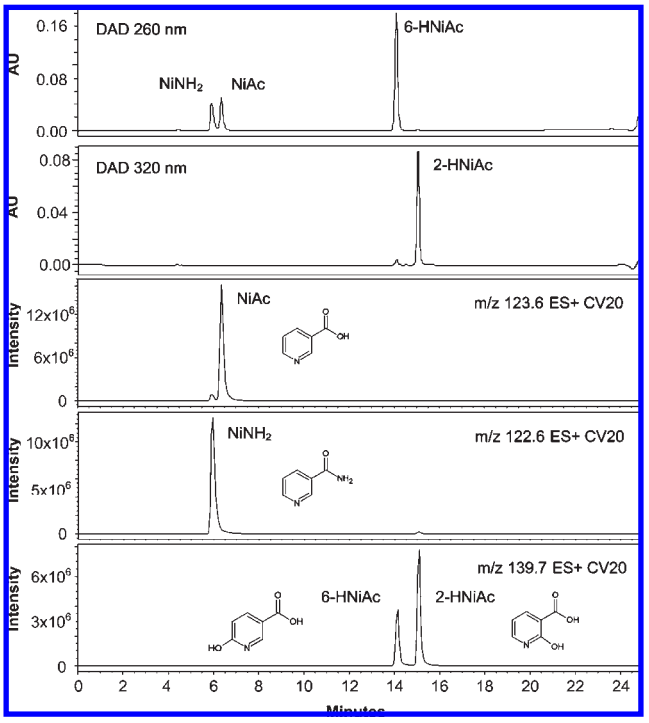

Figure 1. HPLC-DAD and HPLC-MS chromatographic profiles of NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc in standard solution. Chemical structures are shown.

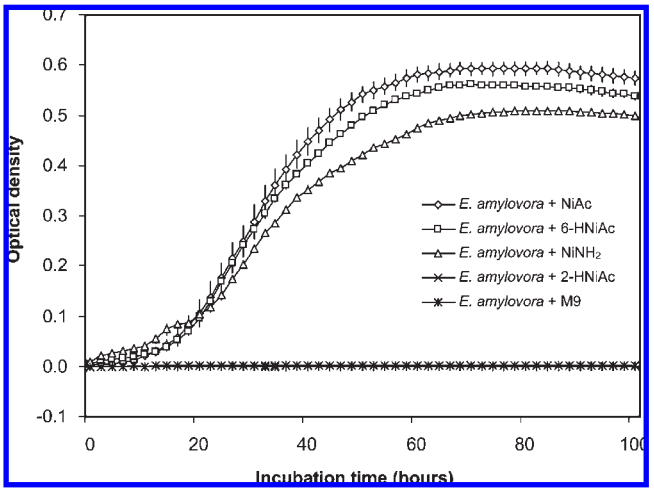

Figure 2. *E. amylovora* CFBP1430 growth in M9 minimal medium + 0.3 mM thiamin−hydrochloride with added 0.8 mM NiAc, 6-HNiAc, and $NiNH_2$, respectively. No growth was assessed in the minimal medium with added 0.08 mM 2-HNiAc or in the nonamended medium (the signals are overlapping at OD = 0.0). An OD = 0.04 corresponds to 10^8 CFU/mL. Values in the graph are the average of four measurements. Standard deviations are shown.

when *E. amylovora* was cultivated in minimal medium supplemented with NiAc, $NiNH_2$, or 6-HNiAc, indicating that each of these compounds can be used efficiently as growth factor in vitro (**Figure 2**). *E. amylovora* was instead unable to grow in the presence of 2-HNiAc as growth factor. In this case, as well as in nonamended medium, the optical density was not different from the blank (noninoculated medium).

Method Validation. A pooling of 15 blossoms per sample was considered to be appropriate to reduce variability due to individual blossoms.

Extraction Recovery. For the validation of the method, NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc recoveries were evaluated. Because of the differences in morphology between apple and pear blossoms, distinct relative recoveries were calculated for apple (cv. Golden Delicious) and pear (cv. Luise Bonne) samples. Extraction recoveries were quantified by calculating percentage recovery of the average of 10 fortified and 10 unfortified samples. Values achieved for each compound are indicated in **Table 2**.

Linearity and Range. Linear calibration curves were obtained for NiAc, $NiNH_2$, 6-HNiAc, and 2-HNiAc in the range 0.08−4.00 mg/L, with coefficients of correlation (R^2) of, respectively, 0.999162, 0.999075, 0.998155, and 0.999897 for MS detection. Calibration curves for NiAc and $NiNH_2$ are shown in **Figure 3**.

Limit of Detection (*LOD*) *and Limit of Quantification* (*LOQ*). LOD and LOQ were experimentally estimated as, respectively, 3 and 10 times the signal-to-noise ratio (S/N).

Values obtained for DAD and MS detection are indicated in **Table 2**.

Repeatability. Instrumental repeatability was calculated from 10 consecutive measurements of the same pear sample (cv. Spadona). Average contents of 16.81 and 1.82 μg/g blossom with relative standard deviations (RSD) of 6.94 and 16.48 were obtained for NiAc and $NiNH_2$, respectively.

Examples of HPLC-MS chromatograms obtained for apple and pear samples are given in **Figures 4** and **5**, respectively. The presence of NiAc and $NiNH_2$ was confirmed by comparing the retention times and UV and MS spectra with those of authentic standard. High-accuracy MS were also obtained by the UPLC-Q-TOF configuration on some apple and pear extracts, further confirming each peak with a MS accuracy of ±2.5 ppm. The nominal mass accuracy achievable with the LC-MS configuration

Table 2. Recovery in Apple and Pear Blossoms and Instrumental LOD and LOQ Values Calculated for DAD and MS Detection[a]

	recovery (%)		DAD		MS	
	apple	pear	LOD	LOQ	LOD	LOQ
NiAc	89.3	81.0	8.0	26.6	4.0	13.2
$NiNH_2$	67.1	50.7	7.8	26.0	8.6	28.8
6-HNiAc	68.2	89.9	2.7	9.1	13.8	45.8
2-HNiAc	94.1	90.5	8.7	29.0	14.8	49.4

[a] Values are expressed in μg/L.

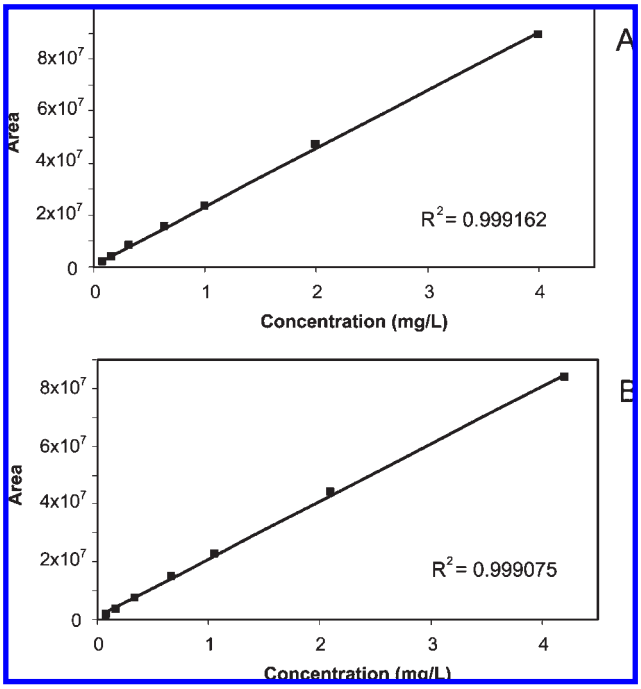

Figure 3. Calibration curves obtained in HPLC-MS for NiAc (**A**) and $NiNH_2$ (**B**).

was found to ensure an adequate selectivity and was chosen as the best approach for the routine method.

Detection and Quantification of Nicotinic Acid and Analogues/Derivates in Apple and Pear Hypanthium. In apple and pear hypanthium, 6-HNiAc and 2-HNiAc contents were found to be below the detection limit of the method. We proceeded therefore to the quantification of NiAc and $NiNH_2$ amounts.

NiAc Quantification. On average, pear samples were found to contain almost 100 times more NiAc than apple samples. Among the apple species, the highest NiAc amount was found in Japanese crab apple (*M. floribunda*) with 0.61 μg/g of blossom. Crab apple (*M. sylvestris*) showed a content equal to 0.25 μg of NiAc/g of blossom. In apple (*M. domestica*) varieties, NiAc content ranged from 0.22 μg/g of blossom in Braeburn to 0.46 μg/g of blossom in Fuji. Intermediate contents were observed in Pinova, Canada Reinette, Stark Delicious, and Golden Delicious with, respectively, 0.23, 0.29, 0.43, and 0.45 μg/g of blossom (**Figure 6A**).

In pear (*P. communis*), NiAc content ranged from 14.15 μg/g of blossom in Spadona 1 to 37.83 μg/g of blossom in Spadona 2. Louise Bonne and Williams 1 showed slightly higher NiAc concentrations that Spadona 1, whereas higher amounts of NiAc were found in Williams 2 (31.87 μg/g of blossom) and Spadona 2 (**Figure 6B**).

$NiNH_2$ Quantification. Contrarily to that observed for NiAc, $NiNH_2$ contents were comparable in apple and pear samples. Among the apple species, crab apple showed the higher $NiNH_2$ content, with 6.04 μg/g of blossom. Japanese crab apple content

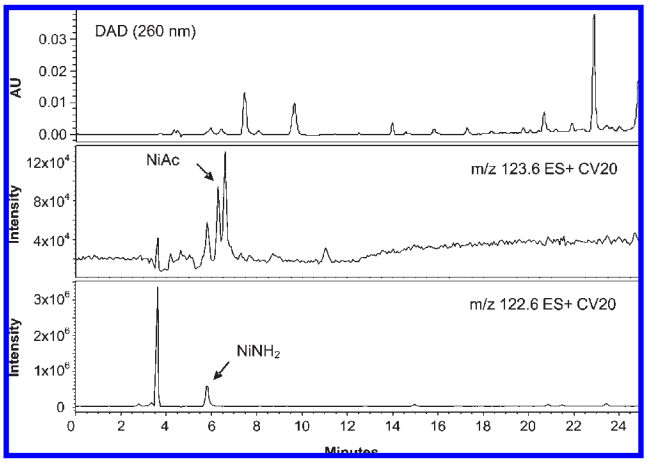

Figure 4. Chromatographic profiles of NiAc and $NiNH_2$ in an apple sample (cv. Golden Delicious).

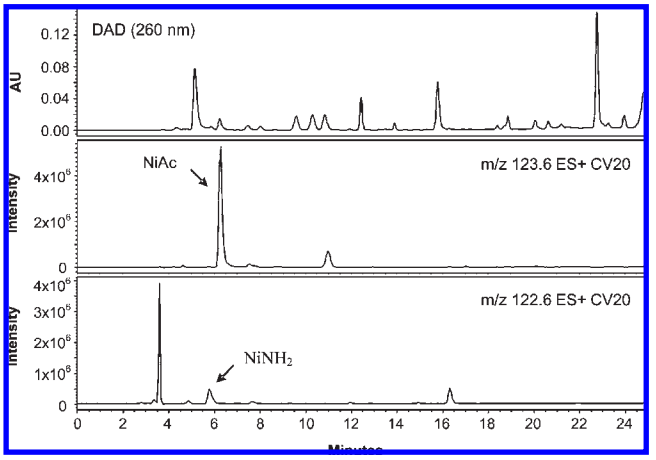

Figure 5. Chromatographic profiles of NiAc and $NiNH_2$ in a pear sample (cv. Luise Bonne).

was found equal to 4.51 μg/g of blossom. In apple varieties, the highest $NiNH_2$ content was observed in Golden Delicious (5.46 μg/g of blossom), whereas the smallest amount was found in Braeburn (2.89 μg/g of blossom). Intermediate values were obtained for Fuji, Pinova, Canada Reinette, and Stark Delicious (**Figure 7A**).

In pear, the smallest $NiNH_2$ content was detected in Spadona 1 (1.39 μg/g of blossom). Intermediate values were found in Louise Bonne, Williams 1, and Williams 2, whereas Spadona 2 showed the highest content (4.64 μg/g of blossom) (**Figure 7B**).

Differences in NiAc and $NiNH_2$ contents observed between samples of the same variety but collected in different places (Spadona 1 and 2, Williams 1 and 2) indicate that environmental conditions found within different orchards may have a stronger effect than purely varietal differences. Notably, Spadona 2, which had higher NiAc and $NiNH_2$ contents, also had larger blossom size compared to Spadona 1 (2.05 vs 1.45 g of blossom weight, respectively) (**Table 1**). Different environmental conditions may also have affected the tree physiological state, contributing to the different nectar composition. Further studies are necessary to investigate the environmental factors affecting the variability of NiAc and $NiNH_2$ contents in apple and pear blossoms.

With this work, we demonstrate for the first time the capability of the fire blight pathogen, *E. amylovora*, to utilize in vitro not only NiAc but also $NiNH_2$ and 6-HNiAc as alternative growth factors. The pathogen was instead unable to use 2-HNiAc.

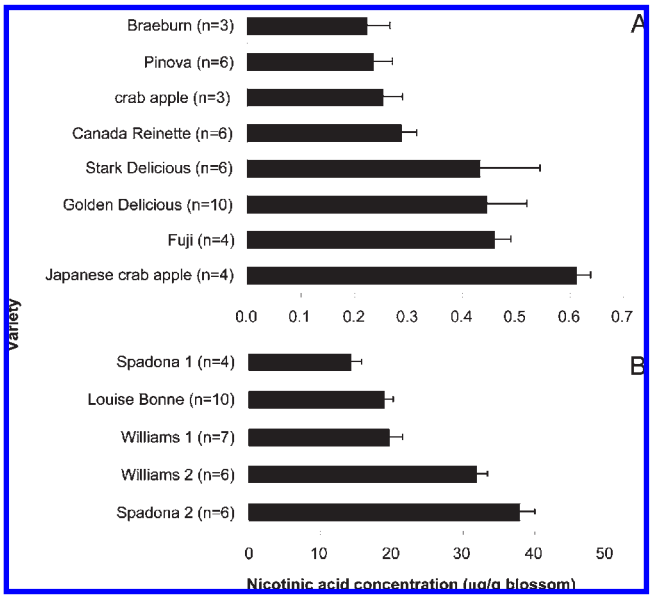

Figure 6. Contents of NiAc in the hypanthia of different varieties of apple (**A**) and pear (**B**). Values represent the mean calculated on the number of samples reported in parentheses, with standard deviation bars.

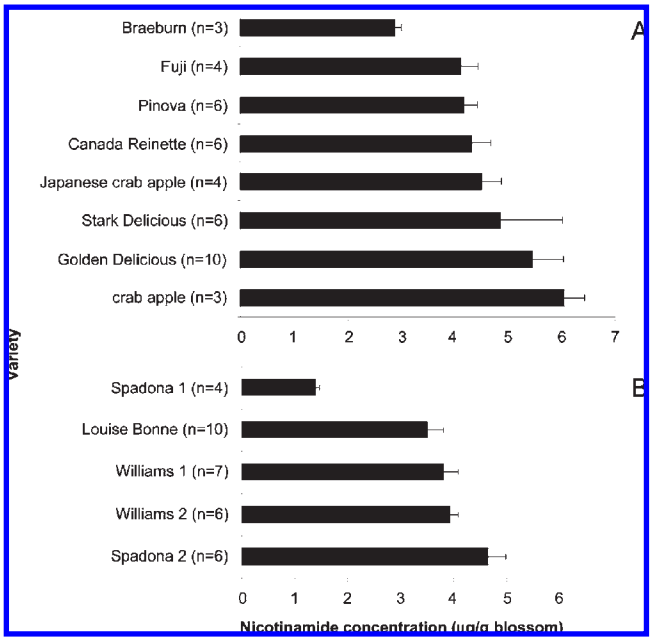

Figure 7. Contents of $NiNH_2$ in the hypanthia of different varieties of apple (**A**) and pear (**B**). Values represent the mean calculated on the number of samples shown in parentheses, with standard deviation bars.

Moreover, by means of an appositely developed HPLC-MS method, we were able to provide the first confirmation of the presence of NiAc and $NiNH_2$ in apple and pear hypanthium and to exclude the presence of 6-HNiAc and 2-HNiAc. A deep knowledge of the chemistry of the infection court of blossom blight is fundamental in understanding the process of establishment of *E. amylovora* and, consequently, for the development or improvement of control strategies at blossom level. NiAc degradation by microbial agents has been recently proposed as new valid approach for the control of fire blight. In light of the results obtained, some consideration must be taken. In pear flowers, NiAc content is about an order of magnitude higher than the content of $NiNH_2$. Therefore, we can hypothesize that NiAc could play a more relevant role than $NiNH_2$ in the pathogen establishment. In apple flowers, instead, $NiNH_2$ content is approximately an order of magnitude higher than the content in NiAc, suggesting a more relevant role for $NiNH_2$ than for NiAc.

Our results, although not sufficiently abundant to perform an appropriate statistical analyses, suggest a different composition of the nectar between apple and pear and, perhaps, among different varieties. Pear is generally considered to be a more susceptible host to fire blight than apple (*34*); therefore, these substantial differences in nectar composition are compatible with a role of NiAc and $NiNH_2$ in the differential susceptibilities toward the pathogen. Further analyses are therefore required to clarify the role played by NiAc and $NiNH_2$ in the establishment of *E. amylovora* in blossoms and in the differential susceptibilities to fire blight.

ABBREVIATIONS USED

NiAc, nicotinic acid; $NiNH_2$, nicotinamide; 6-HNiAc, 6-hydroxynicotinic acid; 2-HNiAc, 2-hydroxynicotinic acid.

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