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Nicotinic acid and nicotinamide on pear and apple flowers are not limiting factors for *Erwinia amylovora* growth when these chemicals are considered in relation to cultivar and flower age

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Summary. Fire blight, caused by *Erwinia amylovora*, is a devastating disease of pear (*Pyrus communis*) and apple (*Malus* × *domestica*) in many areas of the world. The disease is often initiated by epiphytic populations that multiply on flowers and colonize the hypanthia. *In vitro*, *E. amylovora* requires nicotinic acid (NicAc) and/or nicotinamide ($NicNH_2$) as essential growth factors. The amount of NicAc on pear hypanthia was positively correlated with the altitude of the growing site and was inversely correlated with the sum of the maximum temperatures in the 30 days before flowering. The sum of the amounts of NicAc and $NicNH_2$ on the hypanthia was about 6 to 23 times higher in pear, and about 1.2 to 3.5 times higher in apple, than the amounts of NicAc or $NicNH_2$ necessary to support maximum *E. amylovora* growth *in vitro*. No correlation was found between the amounts of NicAc and $NicNH_2$ on the hypanthia of different pear and apple cultivars and at different growth stages and the growth of *E. amylovora* after experimental inoculation. In conclusion, NicAc and $NicNH_2$ are essential for *E. amylovora* growth but the amounts of these chemicals on pear and apple flowers do not limit the establishment of the pathogen when competing bacteria are lacking.

Key words: vitamin B3, hypanthium, fire blight, altitude, *Rosaceae*.

Introduction

Erwinia amylovora causes fire blight, a serious and hard-to-control disease affecting pear and apple almost worldwide (Johnson and Stockwell, 1998; Vanneste, 2000). All of the aerial parts of the tree can be affected, although the disease is often initiated by epiphytic populations of the pathogen growing on the stigmas and hypanthia of the flowers (Thomson, 1986). The stigma exudate and the nectar provide nutrients for the pathogen growth (Buban *et al.*, 2003). Generally, pear is more susceptible to fire blight than apple (Eastgate, 2000). However, pear and apple stigma exudates and nectars have a similar composition in free sugars and free amino acids (Pusey *et al.*, 2008).

Erwinia amylovora requires nicotinic acid (NicAc), nicotinamide ($NicNH_2$) and/or 6-hydroxynicotinic acid (6-HNicAc) as essential growth factors when it is cultured on laboratory minimal media (Starr and Mandel, 1950; Paternoster *et al.*, 2009b). NicAc and $NicNH_2$, but not 6-HNicAc, occur on pear and apple hypanthia. Amounts of NicAc are approximately two orders of magnitude greater on the hypanthia of pear than on apple hypanthia (Paternoster *et al.*, 2009b). Since pear is more susceptible to fire blight and also has a higher amount of NicAc than apple, there may be a correlation between fire blight susceptibility and the amounts of NicAc and $NicNH_2$ on the hypanthium.

The flower growth stage affects the capacity of the flower to support *E. amylovora* growth, with bacterial populations being significantly smaller

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on older flowers (Thomson and Gouk, 2003). To date, however, the amounts of NicAc and $NicNH_2$ have only been assessed on newly opened flowers (Paternoster *et al.*, 2009b), and not on flowers at other growth stages.

The altitude of the growing site affects the production of several metabolites in plants (Spitaler *et al.*, 2006; Ganzera *et al.*, 2008). Similarly, amounts of NicAc and $NicNH_2$ on pear and apple flower hypanthia could also be affected by altitude, but here too no data are available.

In this study, we assessed the amounts of NicAc and $NicNH_2$ on the hypanthia of flowers of several pear and apple cultivars, and on the hypanthia of pear and apple flowers at different growth stages. Two different growing altitudes for each cultivar and each growth stage were considered. We also inoculated *E. amylovora* directly on the hypanthia to determine whether there was a correlation between the amounts of NicAc and $NicNH_2$ on the hypanthia and the capacity of flowers to support pathogen growth.

Materials and methods

Bacterial strain and culture conditions

Erwinia amylovora CFBP 1430 rfm^r (resistant to 100 μg rifampicin mL^{-1}), a spontaneous derivative of *E. amylovora* CFBP 1430 (Paulin and Samson, 1973) with unaltered virulence (Duffy B., unpublished data), was used in the experiments. The strain was routinely cultured on diluted (10%) tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 1.2% agar (Oxoid, Basingstoke, UK) (TSA) at 24°C.

Flower samples

The amounts of NicAc and $NicNH_2$ and the growth of *E. amylovora* were assessed on the hypanthia of pear (*Pyrus communis*) cv. Abbé Fétel, Kaiser, Conference, Louise Bonne and Williams, and apple (*Malus* × *domestica*) cv. Canada Reinette, Fuji, Golden Delicious, Pinova and Stark Delicious. Newly opened flowers, with no dehisced anthers, were collected early in the morning before sunrise from two sites per cultivar located in the Trentino region, one of the most important horticultural areas in northern Italy, at two different altitudes (here designated as low and high) well within the altitudinal range of pear and apple. Altitudes for the pear cultivars spanned from 293 to 987 m above sea level (a.s.l.), and for the apple cultivars from 635 to 998 m a.s.l.

The amounts of NicAc and $NicNH_2$ and the growth of *E. amylovora* were assessed on the flowers of apple cv. Canada Reinette and pear cv. Louise Bonne at three growth stages: that of unopened flowers (the day before they were expected to open), of newly opened flowers and of flowers with dehiscent anthers (three days after opening). These flowers were also collected from plants growing at two sites at two different altitudes (low and high): 656 and 987 m a.s.l. for the pear cv. Louise Bonne, and 878 and 920 m a.s.l. for the apple cv. Canada Reinette.

The trees selected were similar in age (5–15 years for pear and 5–10 years for apple) and received similar agronomic practices (irrigation, fertilization, pruning, etc.). The apple trees were grafted on rootstock M9, and the pear trees on quince.

NicAc and $NicNH_2$ quantification on pear and apple hypanthia

Ten samples, each consisting of 15 flowers, were analyzed for each cultivar, for each growing altitude and for each growth stage. The flowers of each sample were randomly picked from at least three trees. Sample preparation and quantification of chemicals was performed as in Paternoster *et al.*, (2009b). This method was developed to measure NicAc and $NicNH_2$ on the upper layers of the hypanthium, the flower organ consisting of the base of the sepals, petals, and stamens fused together, as is typical of the *Rosaceae*. The petals, stamens, and stigmas were therefore removed from the flowers, and the remaining part was weighed prior to extraction. Extraction consisted of 5 min ultrasonication followed by 10 min extraction in 3.3 mL of 1% formic acid in water, performed three times on the same sample. The three extractions per sample were pooled, adjusted to an exact volume of 10 mL, and filtered through a 0.22 μm filter (Millex-GV, Millipore Corp., Bedford, MA, USA) into HPLC vials. The vials were kept at -20°C until analysis. Mass spectrometric analysis of the samples was carried out. For each compound, values were corrected according to the recovery rates calculated at the time when the

method was developed (Paternoster *et al.*, 2009b) and expressed as μg hypanthium^{-1}.

Erwinia amylovora growth on pear and apple hypanthia

In order to eliminate possible traces of NicAc and $NicNH_2$ from the bacterial inoculum, the pathogen was pre-cultured overnight at 24°C in M9 minimal medium (Sambrook and Russell, 2001) supplemented with 0.3 mM thiamine-hydrochloride (Fluka, Buchs, Switzerland). A bacterial suspension (10^6 cfu mL^{-1}) was obtained by diluting the overnight culture in 0.1 M potassium phosphate buffer (pH 7.0) amended with 0.03% Tween 20. A 10 μL drop of the suspension was then deposed on the hypanthium of intact flowers. Ten flowers per cultivar or per flower age and per altitude were treated. The concentration of the suspension was determined by counting the cfu in a series of serial dilutions cultured on LB agar (Sigma-Aldrich, Steinheim, Germany) amended with the antibiotic rifampicin (100 μg mL^{-1}). The flowers were incubated for 72 h at 18°C under high relative humidity (in closed transparent plastic boxes) and with a 16-h light photoperiod (lateral illumination; intensity: 4000 lux). The population size was then determined using ten flowers per cultivar or flower growth stage per altitude. The hypanthium of each flower was placed in a sterile 2-mL Eppendorf tube containing 1 mL of 0.1 M potassium phosphate buffer. The tubes were vortexed, sonicated for 20 s and vortexed again. The size of the *E. amylovora* population (expressed as cfu flower^{-1}) of each flower was evaluated using the most-probable-number design (Briones and Reichardt, 1999). Microtiter plates (96-well) in which each well contained 180 μL of LB medium supplemented with rifampicin (100 μg mL^{-1}) were used to perform ten-fold dilutions using 20 μL of washing suspension (four wells per dilution). The microtiter plates were covered with a sterile gas-sealing permeable membrane (Breathe-EasyTM, Diversified Biotech, Boston, MA, USA) and incubated for 2 d at 24°C under shaking at 150 rpm. Microtiter plates were then checked for bacterial growth.

Minimum amounts of NicAc and $NicNH_2$ required for _E. amylovora_ growth _in vitro_

An overnight culture of *E. amylovora* CFBP 1430 rfm^r was prepared as above. The culture was diluted with fresh M9 minimal medium supplemented with 0.3 mM thiamine-hydrochloride to reach an optical density of 0.04 at 600 nm (OD_{600}), corresponding to about 10^8 cfu mL^{-1}. The culture was then diluted to a final concentration of 10^6 cfu mL^{-1}. Solutions of NicAc (molecular weight 123.11) and $NicNH_2$ (molecular weight 122.12) (Fluka) at a concentration of 21.5 mg mL^{-1} were prepared in 0.1 M potassium phosphate buffer (pH 7.0). After sterile filtration through a 0.22 μm polycarbonate filter (Millipore), serial dilutions were prepared in M9 minimal medium supplemented with 0.3 mM thiamine-hydrochloride. Aliquots (190 μL) were dispensed aseptically to a 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany) spanning a range of concentrations from 2.15×10^1 to 2.15×10^{-4} μg mL^{-1}. For each dilution, four adjacent wells of the same column were filled with the medium, and the first three wells were inoculated with 10 μL each of the prepared 10^6 cfu mL^{-1} *E. amylovora* suspension (about 10^4 cfu). The non-inoculated well was used as a blank. M9 medium supplemented with 0.3 mM thiamine-hydrochloride was used as the control. After inoculation, the microplate was covered with a sterile gas-sealing permeable membrane (Breathe-EasyTM, Diversified Biotech) and incubated under shaking (150 rpm) at 24°C for 4 d. *E. amylovora* growth was evaluated by OD measurement at 600 nm using a Spectra Fluor Plus microplate reader (Tecan, Crailsheim, Germany) after 36, 48, 60, 72, 84, 96, and 108 h of incubation. To avoid cell sedimentation, before each reading the cultures were lightly shaken for 10 s in the microplate reader. The experiment was repeated once.

Statistical analysis

The amounts of NicAc and $NicNH_2$ on the flowers of the different pear and apple cultivars, and on the pear and apple flowers at the different growth stages, are each presented as the averages ± standard deviation of 10 samples (each sample included 15 flowers). Data were analyzed by analysis of variance (ANOVA) followed by Tukey's HSD post hoc-test (α=0.05).

The relation between the amounts of NicAc on the hypanthia of newly opened pear flowers (in μg g of hypanthium^{-1}) and the sums of the minimum or the maximum daily temperatures (in °C) recorded during the 7, 15, and 30 days before flowering were assessed using Pearson's correlation test (α=0.05).

The average population size of *E. amylovora* (expressed as cfu hypanthium^{-1}) was calculated for each cultivar and for each flower stage, along with the standard deviations of the average. Data were analyzed by ANOVA, followed by Tukey's HSD post hoc-test (α=0.05). Average correlations between *E. amylovora* population size (in cfu hypanthium^{-1}) and the average amount of NicAc and $NicNH_2$ (in μg hypanthium^{-1}) on pear and apple hypanthia were assessed using Pearson's correlation test (α=0.05).

All the statistical analyses were performed with Systat 11 software (Systat Software Inc., Point Richmond, CA, USA).

Results and discussion

The hypanthia of newly opened flowers of the five pear cultivars growing at the high altitude sites had significantly more NicAc than the hypanthia of the same cultivars growing at the low altitude sites (P<0.001) (Figure 1A). Significant differences were found also in 3 of the 5 apple cultivars (Figure 1B). Similar results were reported in Munzuroglu *et al.* (2003) for the content of vitamin C in apricots. Cultivars grown at a high altitude had a significantly higher vitamin C content than the same cultivars grown at the lower altitudes.

The amount of NicAc in pear ranged from 4.300 (cv. Williams) to 2.967 μg hypanthium^{-1} (cv. Louise Bonne) at high altitude, and from 2.059 (cv. Williams) to 0.772 μg hypanthium^{-1} (cv. Kaiser) at low altitude (Figure 1A). In accordance to the data reported in Paternoster *et al.* (2009b), the amount of NicAc on apple was on an average about 45 times lower than that on pear hypanthia, ranging from 0.183 (cv. Canada Reinette) to 0.034 μg hypanthium^{-1} (cv. Stark Delicious and Golden Delicious) at high altitude and from 0.063 (cv. Fuji) to 0.006 μg hypanthium^{-1} (cv. Golden Delicious) at low altitude (Figure 1B). This difference in NicAc between pear and apple hypanthia was much higher than the difference in sugars and amino acids between apple and pear exudates and nectars reported by Pusey *et al.* (2008). On the contrary, the average amounts of $NicNH_2$ on pear and apple hypanthia were similar (P=0.688). The reason for this difference between NicAc and $NicNH_2$ is unclear. In one pear (Williams) and two apple cultivars (Pinova and Stark Delicious) the amount of $NicNH_2$ on the hypanthia was significantly greater in trees at high altitude than in trees grown at low altitude, whereas it was significantly lower in the pear cultivar Abbé Fétel and the apple cv. Fuji (Figure 1C and D). The amount of $NicNH_2$ on pear ranged from 0.681 μg hypanthium^{-1} on Williams to 0.274 μg hypanthium^{-1} on Abbé Fétel at high altitude (Figure 1C). Among the apple cultivars, the amounts of $NicNH_2$ ranged from 0.640 μg hypanthium^{-1} on Stark Delicious at high altitude to 0.257 μg hypanthium^{-1} on Golden Delicious at low altitude (Figure 1D).

Since, as has been mentioned, the amount of NicAc on the hypanthia of pear cultivars was influenced by the altitude of the growing site (Figure 1A) and since temperature is related to altitude, the amounts of NicAc on pear hypanthia were regressed against the sums of the minimum and the maximum daily temperatures recorded before flowering (Table 1). An inverse correlation ($y = -0.32 x + 181.47$) was found between the amounts of NicAc and the sum of the maximum temperatures recorded during the 30 d before flowering (r=0.906, P<0.001; Figure 2). Significant, but less robust correlations were found between the amounts of NicAc on pear hypanthia and the sum of the minimum temperatures, and between the amount of NicAc and the temperatures recorded during 7 and 15 days before flowering (data not shown). A similar tendency was reported by Lee and Kader (2000), who found that levels of vitamin C in citrus fruits were higher when the fruits were grown in a cool environment than when grown in a warm environment.

Erwinia amylovora colonized effectively the hypanthia of all pear and apple cultivars. In newly opened flowers, after 72 h of incubation *E. amylovora* reached numerically similar populations on pear and apple hypanthia (about 10^9 cfu hypanthium^{-1}) (P=0.097) (Figure 1E and F). We can conclude that apple and pear flowers have a similar capacity in supporting *E. amylovora* growth and therefore that the different susceptibility to fire blight between these species cannot be attributed to the considerable differences in the amount of NicAc found on their hypanthia. The altitude of the growing sites did not statistically influence the population size of *E. amylovora* on pear (P=0.295) or apple (P=0.635), except for the pear cultivar

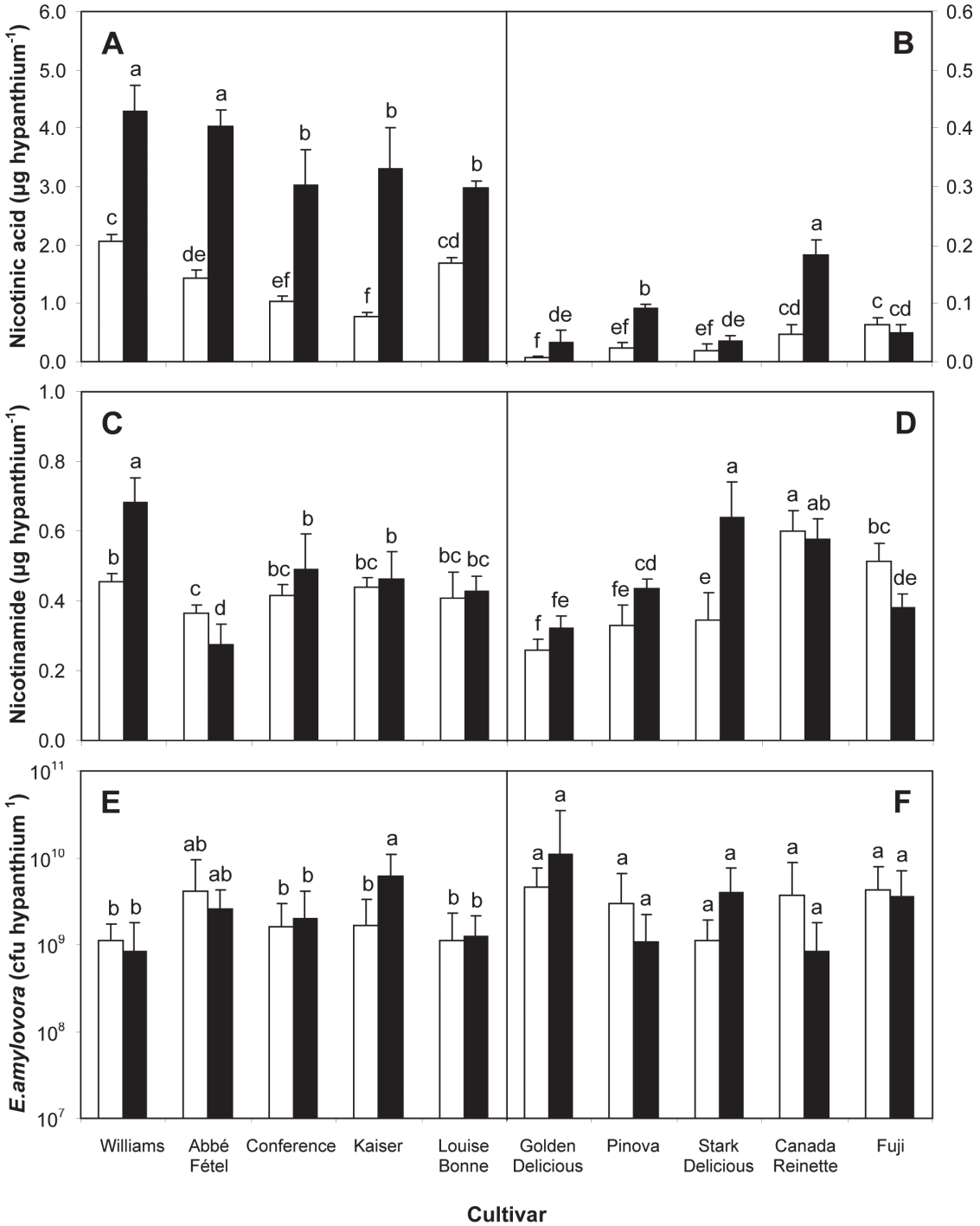

Figure 1. Amounts of nicotinic acid (A, B), nicotinamide (C, D) and *Erwinia amylovora* population (E, F) on hypanthia of newly opened flowers of pear (A, C, E) and apple (B, D, F) cultivars at low (white bars) and high altitude sites (black bars). Flowers from at least three trees of five different cultivars each growing at two altitudes were used. Each value represents the mean of 10 samples (one sample = 15 newly opened flowers) (A, B, C, D). Flower hypanthia were mechanically inoculated with about 10^4 cfu of *E. amylovora* and each value represents the mean of 10 flowers from at least three trees (E, F). Vertical bars represent the standard deviation. Bars with a different letter are significantly different according to ANOVA and Tukey's HSD post hoc-test (α=0.05)

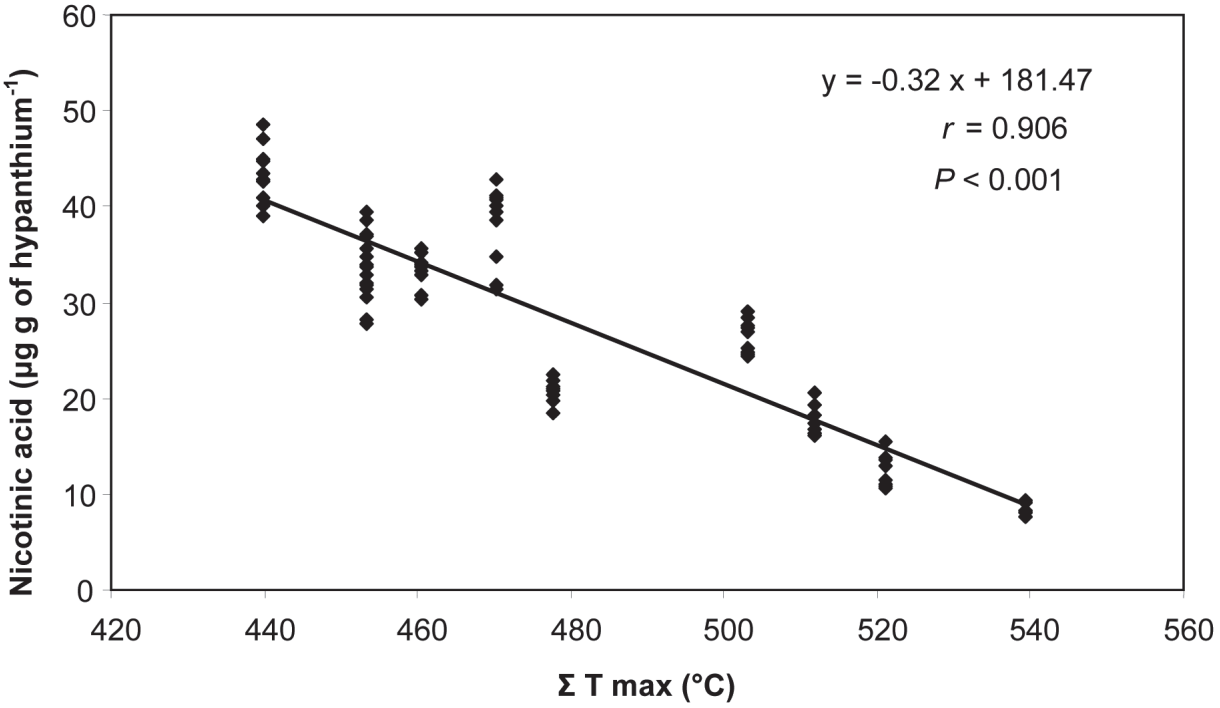

Figure 2. Relation between the amount of nicotinic acid (expressed as µg g of hypanthium^{-1}) on newly opened pear flowers and the sum of the maximum temperatures (Σ T max) (°C) recorded during the 30 days before flowering. For additional information see the caption of Figure 1 and the table 1.

Kaiser, where the *E. amylovora* population was significantly higher at the high altitude (Figure 1E). The correlations between the *E. amylovora* population and the amounts of NicAc and $NicNH_2$ were not significant for either pear or apple (data not shown).

The influence of the flower growth stage (unopened, newly opened, and with dehiscent anthers) on the amounts of NicAc and $NicNH_2$ was studied in the pear cv. Louise Bonne, and in the apple cv. Canada Reinette. In pear the amounts of NicAc in the dehiscent anther stage were two to three times higher than those in the unopened and newly opened stages, irrespective of the altitude, but in apple the amounts of NicAc were about five times higher at the low altitude. The amounts of $NicNH_2$ were also higher at the dehiscent anther stage than at the unopened stage. Differences for $NicNH_2$ were, however, smaller than those for NicAc and reached statistical significance only in pear at high altitude, and in apple at low altitude (Figure 3C and D).

Erwinia amylovora colonized effectively pear cv. Louise Bonne and apple cv. Canada Reinette hypanthia at all three growth stages. Altitude did not influence the *E. amylovora* population on pear and apple flowers (P=0.431 and P=0.074 respectively) (Figure 3E and F). Flowers at the unopened and newly opened stages supported the same *E. amylovora* growth in pear and apple ($1–5 \times 10^9$ cfu hypanthium^{-1}). However, at the dehiscent anther stage apple flowers supported about 10–20 times lower *E. amylovora* growth, whereas this reduction did not occur on pear. This difference was statistically significant at low altitude (Figure 3F). The reduction in *E. amylovora* growth at the dehiscent anther stage was not correlated with the amount of NicAc and $NicNH_2$ on the hypanthium (data not shown). A similar effect of the age of apple flowers on the growth of *E. amylovora* was found by Thomson and Gouk (2003).

NicAc or $NicNH_2$ was required for *E. amylovora* growth in M9 minimal medium. Maximal growth, corresponding to about OD_{600}=0.95 after 72 h of incubation and to about OD_{600}=1.40 after 108 h of incubation, was achieved by adding 0.215 µg mL^{-1} of NicAc or $NicNH_2$. Growth was not improved when NicAc or $NicNH_2$ was added

Table 1. Flower samples analysed as shown in Figure 2.

Variety	Growth site altitude	Elevation[a] (m)	Mean sample weight[b] (g ± SD[c])	Σ T max (°C) 30 days[d]
Williams	Low	293	1.20 ± 0.06	503.2
Williams	High	965	1.60 ± 0.07	470.2
Abate Fetel	Low	293	1.24 ± 0.12	511.9
Abate Fetel	High	920	1.36 ± 0.12	439.9
Conference	Low	293	1.22 ± 0.05	521.0
Conference	High	920	1.40 ± 0.20	453.4
Kaiser	Low	293	1.35 ± 0.04	539.3
Kaiser	High	920	1.48 ± 0.07	453.4
Louise Bonne	Low	645	1.19 ± 0.05	477.7
Louise Bonne	High	987	1.32 ± 0.04	460.5
Golden Delicious	Low	706	1.19 ± 0.07	495.7
Golden Delicious	High	878	1.20 ± 0.07	486.7
Pinova	Low	712	1.08 ± 0.05	502.3
Pinova	High	998	1.22 ± 0.07	524.8
Stark Delicious	Low	652	0.94 ± 0.04	514.9
Stark Delicious	High	719	1.42 ± 0.11	510.7
Canada Reinette	Low	878	1.72 ± 0.06	507.1
Canada Reinette	High	920	1.66 ± 0.05	467.7
Fuji	Low	630	1.14 ± 0.08	516.3
Fuji	High	920	1.12 ± 0.06	449.1

[a] Above sea level.
[b] Each sample consisted of 15 flowers.
[c] Standard deviation.
[d] The meteorological data were obtained from the IASMA meteorological office: http://meteo.iasma.it/meteo/datimeteo/ricercadati.php. For each site, we considered the data of the climatic station more closed to the growing site. Daily T° max measured at 2 m from the soil was obtained from the database and used to calculate the Σ T max (°C) of the 30 days before flowering.

at concentrations 10 or 100 times higher (Figure 4). The total amount of the growth factors available for *E. amylovora* growth on the hypanthium is equivalent to the sum of NicAc and $NicNH_2$. In pear, this amount ranged from 1.210 to 4.981 μg hypanthium^{-1} (Figure 1A–C), which was 6 to 23 times higher than the amount giving maximal growth *in vitro* (0.215 μg mL^{-1}), whereas in apple this amount ranged from 0.264 to 0.761 μg hypanthium^{-1} (Figure 1B–D), which was 1.2 to 3.5 times higher. We can therefore conclude that while NicAc and $NicNH_2$ are essential for *E. amylovora* multiplication, they are not limiting factors in the establishment of the pathogen on apple and pear flowers. However, as described in Paternoster *et al.* (2009a), micro-organisms that degrade NicAc and $NicNH_2$ can be used to reduce the availability of these essential compounds on the hypanthium and therefore to control *E. amylovora* growth.

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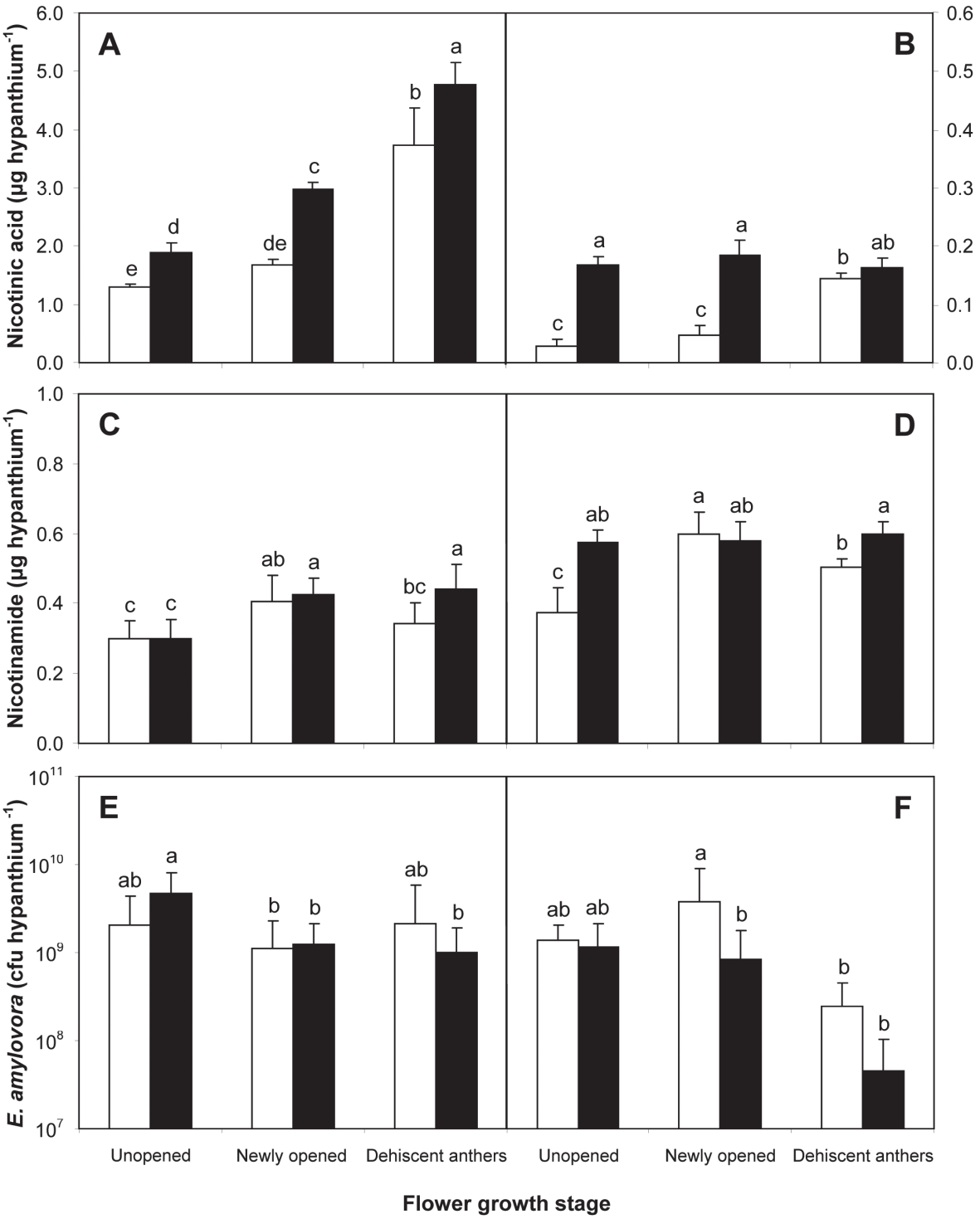

Figure 3. Influence of the flower growth stage in pear cv. Louise Bonne (A, C, E) and apple cv. Canada Reinette (B, D, F) on the amount of nicotinic acid (A, B), and of nicotinamide (C, D) on the hypanthium and on the *Erwinia amylovora* population (E, F). Flowers were collected from trees growing at low (white bars) and high altitudes (black bars). Flowers of at least three trees were used for each growth stage and each altitude. For additional information see the caption of Figure 1.

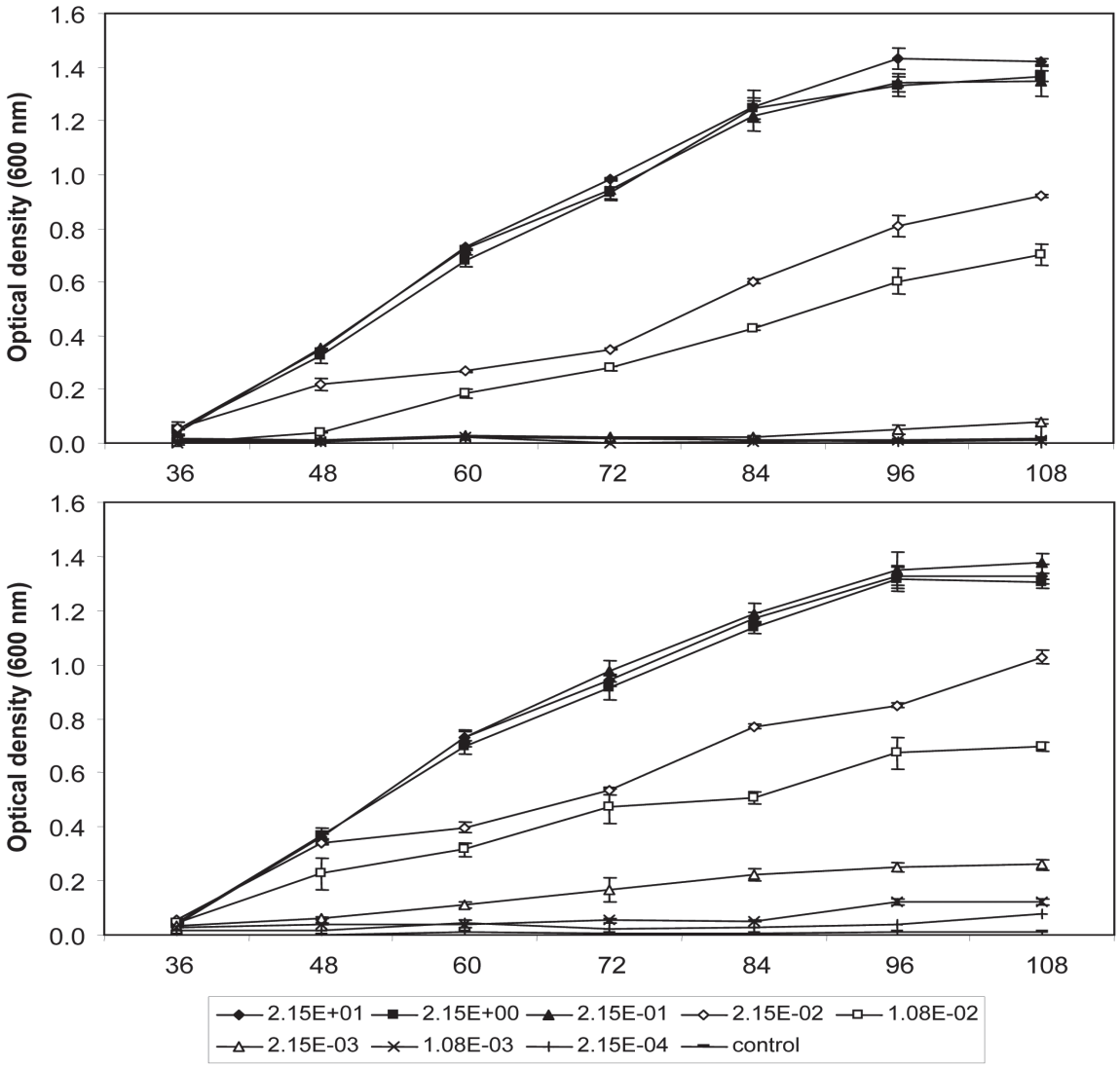

Figure 4. Growth of *Erwinia amylovora* in M9 minimal medium supplemented with 0.3 mM thiamine-hydrochloride and amended with varying amounts (μg mL^{-1}) of nicotinic acid (A) and nicotinamide (B) at 48, 54, 72, 78, 96 and 108 h after inoculation. Each value is the average of 3 replications. Error bars represent the standard deviations of the means. The experiment was repeated once with similar results.

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