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Improved resistance to disease and mites in strawberry, through the use of acibenzolar-S-methyl and harpin to enhance photosynthesis and phenolic metabolism

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Abstract The objective of this study was to evaluate the effect of acibenzolar-S-methyl (ASM) and harpin protein, applied pre-harvest, in the induction of resistance in strawberry to pathogens and mites. The experiment was conducted in a greenhouse, in a randomized block design with three replications. Treatments with harpin protein and ASM were established through biweekly applications of these elicitors in plants of strawberry, from 60 to 120 days after transplanting of seedlings. The application of ASM and harpin protein induced resistance in the strawberry plants, resulting in an increase in phenylalanine ammonia-lyase activity and total phenol production, while reducing leaf blight and grey mould. The application of harpin protein also caused a reduction in mite damage, while increasing photosynthetic rate and the production of marketable fruits. The elicitors ASM and harpin, applied pre-harvest, can be used as part of the integrated pest management of diseases and pests of strawberry.

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1 Introduction

Induced resistance (IR) can lead to the direct activation of defences, but can also lead to the priming of cells, resulting in stronger elicitation of those or other, defences, following pathogen attack. Plant infection by a necrotising pathogen, colonisation of the roots by certain beneficial microbes, or after treatment with cell wall fragments, plant extracts and synthetic chemicals can induce resistance to subsequent pathogen attack, both locally and systemically (Goellner and Conrath 2008; Walters and Fountaine 2009). Many plants establish a unique physiological situation that is called the 'primed' state of the plant. In the primed condition, plants are able to 'recall' the previous infection, root colonisation or chemical treatment. As a consequence, primed plants respond more rapidly and/or effectively when re-exposed to biotic or abiotic stress (Goellner and Conrath 2008).

In broad terms, induced resistance can be split into two main types: systemic acquired resistance (SAR) against pathogens, and induced systemic resistance (ISR) against insects. SAR can be induced by a variety of agents, including necrotizing pathogens and certain chemicals [e.g. acibenzolar-S-methyl (ASM)], and is mediated by a salicylic acid-dependent process (Spoel

and Dong 2012). ISR develops as a result of the colonization of plant roots by certain strains of plant growth-promoting rhizobacteria or biological activators such as harpin protein, and is mediated by a jasmonate- and ethylene-sensitive pathways (Spoel and Dong 2012).

The first chemical resistance activator, Probenazole, was registered in Japan as Oryzemate in 1975, and since then many other chemical and biological activators have been developed, including: ASM, registered as Bion and Actigard (Syngenta), Milsana (*Reynoutria sachalinensis* extract; KHH BioScience), Elexa (chitosan; SafeScience) and Messenger or ProAct (harpin protein; Plant Health Care) (Walters et al. 2013).

Both the ASM and the harpin protein are compounds that are considered of low environmental impact and do not present a risk to human and animal health. The harpin protein and ASM have been effective in controlling several plant diseases (Danner et al. 2008; Mazaro et al. 2008; Alamino et al. 2013). The harpin protein, originally isolated from *Erwinia amylovora* (Wei et al. 1992), and the harpin αβ protein, consisting of harpin protein fractions derived from naturally occurring bacteria, are produced commercially in transformed *Escherichia coli* K-12, that receive the DNA fragment encoding for the protein of interest (United States Environmental Protection Agency 2002; 2005). The harpin proteins have the capacity to activate two plant defence routes, the synthesis of salicylic acid and jasmonic/ethylene acid, which are the signalling molecules for the defence against pathogens and insects, respectively (Dong et al. 1999, 2004; Clarke et al. 2005). In turn, the ASM is an analogue of salicylic acid, which acts in the induction of SAR activating the expression of defence genes against fungi and bacteria, but having no effect against insect (Durrant and Dong 2004; Jalali et al. 2006). This elicitor is released for commercial use in Brazil, for foliar applications in cotton, potato, cocoa, citrus, beans, melon and tomato (Anvisa 2015a).

Widely consumed in natural or processed form, the strawberry has one of the highest rates of pesticide contamination of all the fruits. In the year 2012, 59.2 % of the fruit samples analysed by the National Agency of Sanitary Surveillance of Brazil contained fungicide and/or insecticide residue either not authorized for the inspected culture and/or of a level above those permitted (Anvisa 2015b). The European Union has a short and medium term policy to achieve significant reductions in the use of pesticide in agricultural products (Hillocks 2012). Recently, the Brazilian Senate approves a project that encourages the research, production and use of non-synthetic pesticides for the control of pests and diseases and established the National Policy in Support of Pesticides and Related of Low Danger (Brazilian Senate 2015).

Therefore, there is an urgent need for the establishment of additional methods in plant pest and disease management. The IR in plants is a new approach to the control of pests and diseases and has the prospect of providing a durable and broad spectrum control (Walters et al. 2013).

In this context, the objective of this work was to evaluate the effectiveness of harpin protein and acibenzolar-S-methyl, applied pre-harvest, at inducing resistance in the strawberry cv. 'Camarosa'.

2 Materials and methods

The experiment was carried out in a greenhouse, with controlled temperature (13–28 °C), located at the Technological Federal University of Paraná, Pato Branco, Brazil (26°11′50″S, 52°41′26″W, altitude 820 m).

The strawberry seedlings (*Fragaria* x *ananassa* Duchesne ex Rozier) of the Camarosa cultivar were imported from Chile and cultivated in pots with a volume of 4.5 L, which contained substrate that consisted of a mixture of soil, vermicompost and vermiculite (6:3:1). The substrate had the following chemical characteristics: 73.70 g L^{-1} of organic material; 0.09 g L^{-1} P; 1.09 g L^{-1} K; 1.16 g L^{-1} Ca; 0.53 g L^{-1} Mg; base saturation = 77.2 %; cation exchange capacity = 16.80; and pH (in CaCl2) = 5.20. During the cultivation of the strawberries, sprinkler irrigation was performed daily for a period of fifteen minutes.

Before the application of the elicitors, the fungicides Azoxistrobin (0.17 g L^{-1}) and Thiophanate methyl (0.70 g L^{-1}) were applied at 15 and 33 days after transplantation, respectively, with the objective of maintaining the plants health and vigourous. Further to this, an application of the insecticide β-cyfluthrin + Imidacloprid (2 mL L^{-1}) was applied 111 days after transplantation to control aphids.

The treatments with the elicitors harpin protein, harpin αβ protein and ASM were established by applying the commercial products Messenger (3 % ai) at a dose of 0.75 g L^{-1}, ProAct (1 % ai) at a dose of 2.50 g L^{-1} and Bion 500WG at a dose of 0.0005 kg^{-1}, respectively. Five sprays of the elicitors, with 15-day intervals between each, were performed from 60 to 120 days after transplantation. In addition to this, a spray treatment was applied containing distilled water to act as a control treatment.

Twenty-four hours after the second application of the elicitors (75 days after transplantation), we proceeded with the inoculation of the fungus *Botrytis cinerea* Pers ex Fr (10^8 spores L^{-1}), the causal agent of floral abortion and gray mould in strawberries. The inoculum was isolated from diseased fruits and cultivated in a culture medium of potato-dextrose agar and then kept for 15 days in a BOD incubator at 25 °C, with a photoperiod of 12 h. The concentration of spores was set in suspension using a hemacytometer. The mites occurrence was natural, without inoculation.

Randomized blocks were adopted for the experimental design, with three replicates of sixteen strawberry plants for each treatment, but different number of plants were used to each evaluation. The severity of leaf blight, caused by the fungus *Dendrophoma obscurans* (Ell. & Ev. H.W. Anderson), was evaluated in all leaves of five plants of each experimental unit, at intervals of 10 days, from 68 to 118 days after transplantation, comprising six evaluations in total. To determine the severity indexes, we adopted the specific diagrammatic scale for evaluating the disease (Mazaro et al. 2006). The data were used to calculate the area under the disease progress curve: $AUDPC = \sum\{[(Y_i + Y_{i+1}/2) \times [(T_{i+1} - T_i)]]\}$, where Y_i = severity at the nineth observation and T_i = age (days) of the plant at the nineth observation. For the same plants used for the severity evaluation of the leaf blight, an evaluation was made of mite incidence 108 days after transplanting, by counting plants that presented signs of disease caused by mites (tan or white stains and the formation of web on the underside of the leafs).

The healthy fruits were harvested twice per week from 68 to 130 days after transplantation, totalling 19 samples, in six plants of each experimental unit. The marketable fruits were counted and weighed to obtain the number of fruits per plant, the weight of fruits per plant and the mean weight of the fruits. Further to this, we also evaluated the incidence of gray mould (percentage of fruits with rot in relation to the total fruits collected). For the same plants, assessments were made of the percentage of aborted flowers, every 10 days, from 78 to 108 days after transplantation, totalling four evaluations.

Forty-eight hours after the last application of the treatments (122 days after transplantation), evaluation of the gas exchanges were made using a portable infrared gas analyser (model LI-6400*xt*, LI-COR Inc., Lincoln, NE, USA). The microclimate conditions in the measuring chamber were 26 °C, 1300 photons μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), relative air humidity of 61 % and 400 μmol CO_2 mol^{-1}. Three fully expanded leaves were evaluated per plant, with three plants evaluated per experimental unit. The reading of each leaf was made in triplicate.

For the biochemical analyses five leaves were collected from five plants per experimental unit at 24 before and 24, 48 and 72 h after the first and second application of the elicitors, 60 and 75 days after transplantation, respectively. The collected leaves were wrapped in aluminium foil, then immediately frozen in liquid nitrogen and then stored in a freezer (at −40 °C). These leaves were submitted for the quantification of total proteins, using the method proposed by Bradford (1976), total phenols, by the method described by Nozella (2001), and phenylalanine ammonia lyase enzyme activity (EC 4.3.1.5), by the methodology described by Rodrigues et al. (2006). For the biochemical analysis, the measurement reading were made in triplicate.

The data was subjected to the Shapiro–Wilk normality test, followed by an analysis of variance and a Scott-Knott test ($p \leq 0.05$) to compare the means. In addition to this, we applied a t test ($p \leq 0.05$) to compare the activity values of PAL and for the total phenol content between the first and second application of elicitors.

3 Results

There was a significant reduction in the area under the progress curve for leaf blight severity in leaves, for the percentage of aborted flowers and for the incidence of grey mould in fruits, in strawberries, cv. ‘Camarosa’,

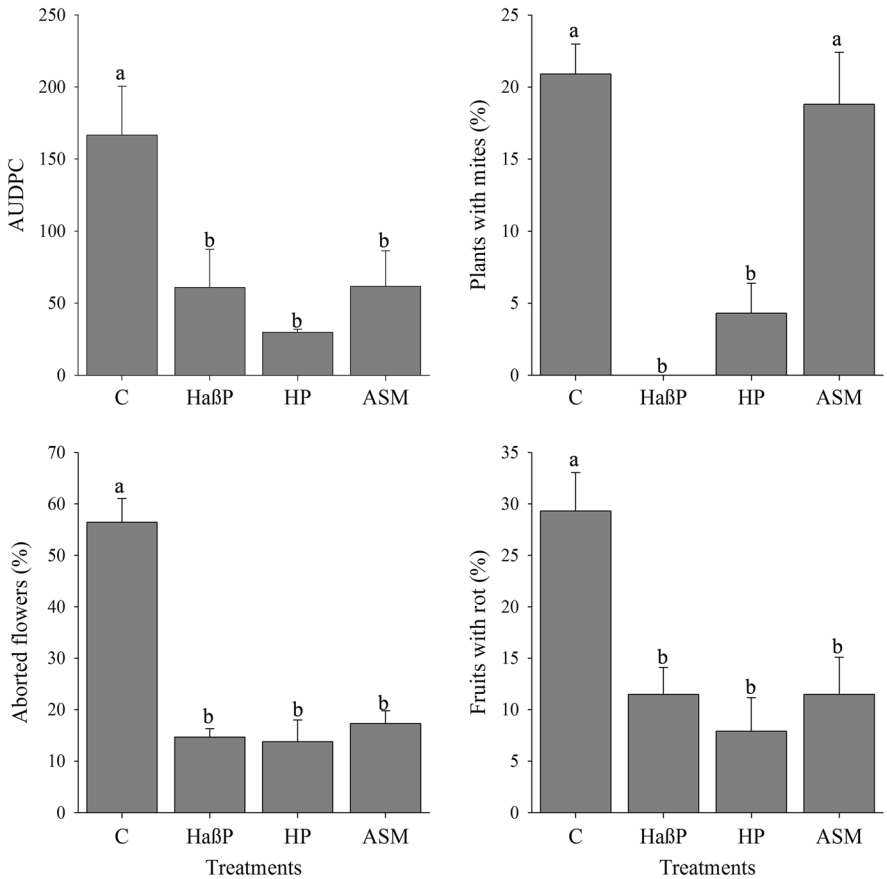

Fig. 1 Area under the disease progress curve (AUDPC) for severity of leaf blight, percentage of plants with mites, percentage of aborted flowers and percentage of fruit with rot caused by *Botrytis cinerea*, in strawberry, cv. 'Camarosa', treated with harpin αβ protein (HαβP), harpin protein (HP) or acibenzolar-S-methyl (ASM). The *bars* represent the standard error of the means. Means followed by *different letters* are statistically different by the Scott-Knott test ($p \leq 0.05$). Data normality was confirmed for all the variables using the Shapiro–Wilk test ($p \leq 0.05$)

treated with harpin αβ protein, harpin protein and ASM when compared with the control treatment (Fig. 1). The incidence of mites on the leaves of the strawberries, cv. 'Camarosa', was reduced significantly through the use of the harpin proteins, especially the harpin αβ protein, which was capable of promoting the absence of signs of mite attack. However, ASM was not effective in controlling the mites, since the incidence of the mites in the plants elicited with ASM did not differ from that observed for the control plants.

The number of fruits and the weight of marketable fruits per strawberry plant was higher in the treatments with harpin, which differed significantly from the control and the elicitor ASM (Fig. 2). The mean weight of marketable fruits was not significantly different between the treatments. There was a higher rate of net CO_2 assimilation in the strawberries treated with harpin αβ, the second highest rate was caused by harpin, these rates differed significantly from those treated with the ASM treatment and the control.

The total protein content was less in strawberries treated with the two harpins, 24 h after the first application of the elicitors (Fig. 3). For the other periods there were no significant differences between the treatments. A higher activity of the enzyme phenylalanine ammonia lyase (PAL) was observed in strawberries treated with the harpins and ASM elicitors, which differed significantly from the control, for all the evaluation periods (24, 48 and 72 h) after the first and second application of the elicitors. The total phenol content showed an increase only after 72 h after the first and second sprays of the harpin and ASM elicitors, which differed significantly from the control and harpin αβ. It should be stressed that there was no significant difference between the protein contents, PAL and total phenols in the strawberries, 24 h before the first and second application of the elicitors.

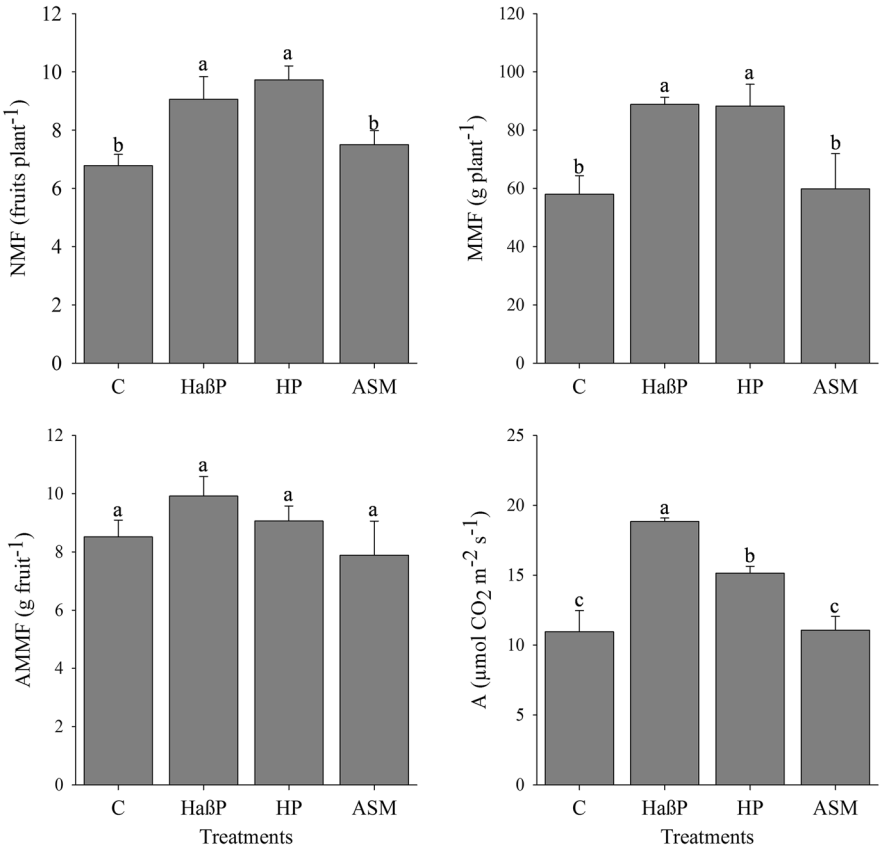

Fig. 2 Number of marketable fruits per plant (NMF), weight of marketable fruits per plant (WMF), mean weight of marketable fruits (MWMF) and photosynthesis (A) in strawberry, cv. 'Camarosa', treated with harpin αβ protein (HαβP), harpin protein (HP) or acibenzolar-S-methyl (ASM). The *bars* represent the standard error of the means. Means followed by *different letters* are statistically different by the Scott-Knott test ($p \leq 0.05$). Data normality was confirmed for all the variables using the Shapiro–Wilk test ($p \leq 0.05$)

4 Discussion

The results for AUDPC, percentage of aborted flowers and for the incidence of grey mould in fruits, in strawberries, cv. 'Camarosa', demonstrate that harpin and ASM were efficient at controlling the fungi *D. obscurans* and *B. cinerea* in the studied strawberry cultivar. Other studies have demonstrated control of plant diseases with the use of these elicitors. In pepper, ASM reduced signs of disease caused by *Phytophthora capsici* by up to 45 %, compared with the control (Baysal et al. 2005). Terry and Joyce (2000) demonstrated that a single or multiple pre-harvest foliar treatment with acibenzolar at anthesis delayed by about 1.2-fold the development of grey mould disease on harvested strawberry cvs. Andana and Elsanta fruit held at 5 °C. This delay was equivalent to a 15–20 % increase in storage life of the fruit. Further to this, Tubajika et al. (2007) observed a reduction of up to 74 % in the incidence of the bacteria *Xylella fastidiosa* in grapevines through the use of harpin protein.

In addition, the incidence of mites on the leaves of the strawberries, cv. 'Camarosa', was reduced significantly through the use of the harpin proteins, especially the harpin αβ protein. The ability of harpin to control phytopathogens and insects, is due to the simultaneous activation of the salicylic acid and jasmonic/ethylene acid defence pathways, whereas ASM doses do not promote the activation of the second pathway and, therefore, has no effect as an resistance elicitor against insects (Durrant and Dong 2004). Jasmonic acid and ethylene are hormones that play a key role in the induction of the immune response of plants independent of salicylic acid, by promoting resistance against pathogen and herbivore attack (Howe and Jander 2008). Dong et al. (1999) found that *Arabidopsis thaliana* mutants for the *nahG* and *NIM1* genes and, therefore, deficient in salicylic acid accumulation and in their response to such molecules, were not influenced by the application of this signaller, observing the absence of the hypersensitive response and of the expression of systemic acquired resistance. However, the plants also showed a

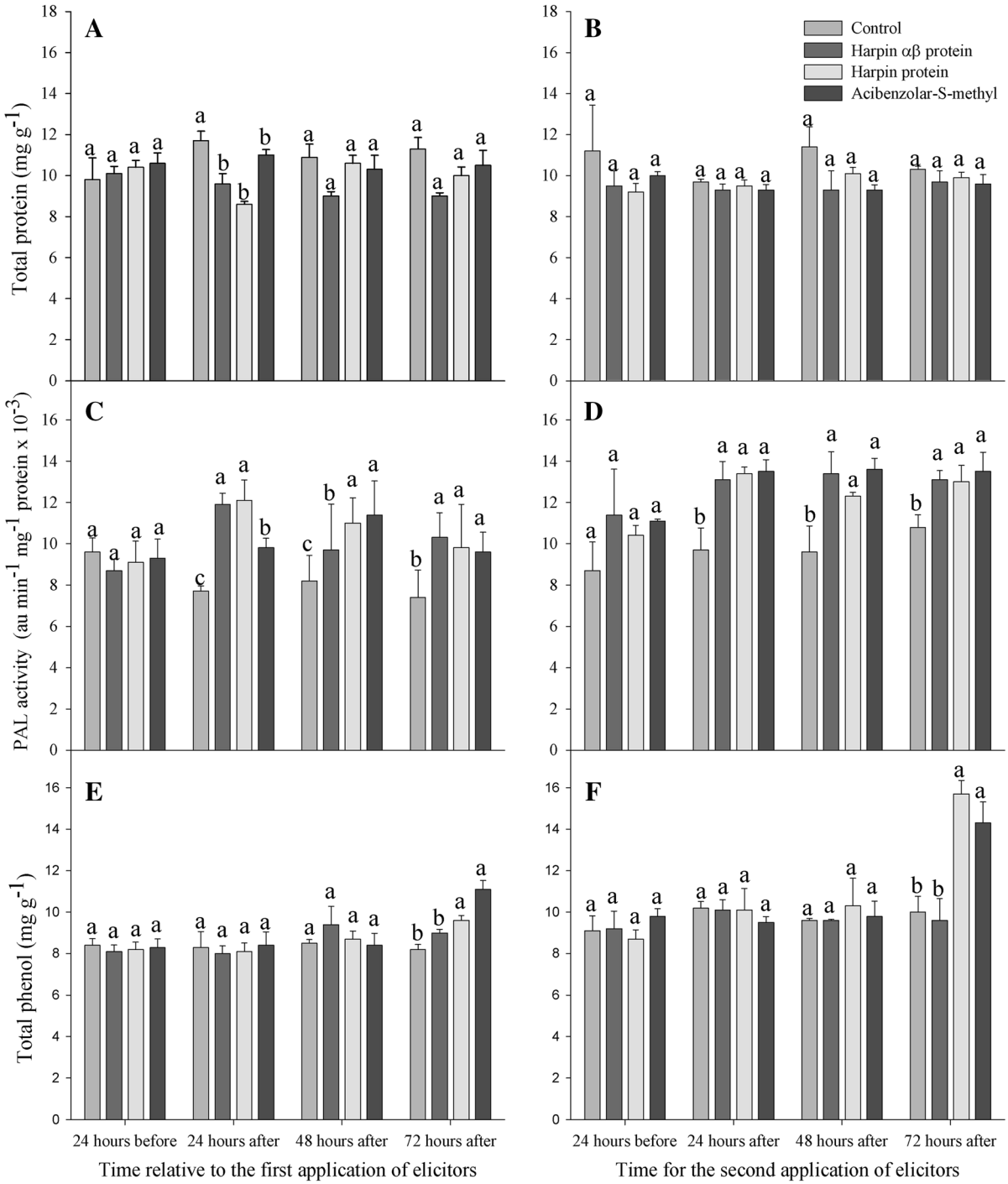

Fig. 3 Total protein content (**a** and **b**) activity of the enzyme phenylalanine ammonia lyase (PAL) (**c** and **d**) and the total phenol content (**e** and **f**) in strawberry, cv. 'Camarosa', treated with harpin αβ protein (HαβP), harpin protein (HP) or acibenzolar-S-methyl (ASM). The *bars* represent the standard error of the means. Means followed by *different letters* are statistically different by the Scott-Knott test ($p \leq 0.05$). Data normality was confirmed for all the variables using the Shapiro–Wilk test ($p \leq 0.05$). The fungus *Botrytis cinerea* was inoculated on the strawberries 24 h after the second application of elicitors

response to methyl jasmonate and ethylene, indicating that other genes controlled this signalling pathway. In studies conducted by Dong et al. (2004) a marked reduction in the incidence of aphids was observed through the application harpin in *A. thaliana*. Tubajika et al. (2007) found that the application of harpin, while reducing infections caused by *Xylella fastidiosa* in grapevine, also reduced the population of the vector that transmits it (*Homalodisca coagulata*).

The harpin protein increases the production of biomass (Tubajika et al. 2007; Li et al. 2014), possibly due to the greater photosynthetic activity, as was verified in the results of the present study, explaining the greater fruit production (NMF and WMF) in the plants that we found to HαβP and HP treatments. In addition to this, the impairment of the leaf tissue through the impact of the leaf blight and mites may have contributed to the reduction of the photosynthetic activity in the control plants and those treated with ASM.

As showed, the total protein content was less in strawberries treated with the HαβP and HP, 24 h after the first application of the elicitors. For the other periods there were no significant differences between the treatments. Weidhase et al. (1987) found that amino acids released by the degradation of photosynthetic proteins (such as the rubisco enzyme) were reused in defence protein synthesis. However, due the ability of the plants to divide nutrients between growth and defence, the costs involved may be visible only under conditions where plant nutrients are limiting (Heil 2001). In this experiment, even though fertilization was not performed, the substrate used had good fertility and apparently did not affect the supply of nutrients to the strawberries. This may have contributed to not limiting the capacity of the plants in maintaining the proteins while still producing the enzymes and compounds necessary for defence in response to the application of the elicitors.

The higher activity of the PAL enzyme was observed in strawberries treated with the harpins and ASM elicitors 24, 48 and 72 h after the first and second application of the elicitors. The total phenol content showed an increase only after 72 h after the first and second sprays of the harpin and ASM elicitors (Fig. 3). Corroborating with our results, other authors using ASM and harpin elicitors have demonstrated that the increase in PAL activity resulted in the increase in total phenol content and reduced the signs of the diseases caused by inoculated fungi (Baysal et al. 2005; Danner et al. 2008; Lin et al. 2009). It was found that the greatest increases in PAL were observed in plants inoculated with fungus (Baysal et al. 2005; Lin et al. 2009), compared with non-inoculated plants. This indicates that there is a greater defence induction when there is necessity, improving the cost/benefit of resistance induction.

In the present study, the activity of the PAL and the total phenol content after the first application of the elicitors was significantly (*t* test, $p \leq 0.05$) lower in relation to the results found after the second application, including the control. The exceptions were the PAL activity in the control 24 h before spraying and the total phenol content for the harpin treatment 24 h before spraying and for harpin αβ 48 h after spraying, for which there was no significant difference between the first and the second applications of the treatments. This indicates that the inoculation of *B. cinerea*, which was performed only 24 h after the second application of the elicitors, promoted greater defence activation, probably due the physiological situation that is called the 'primed' state where the plants are able to 'recall' the previous chemical treatment. As a consequence, primed plants respond more rapidly and/or effectively when re-exposed to biotic or abiotic stress (Goellner and Conrath 2008). Further to this, it indicates that there was a cumulative effect of the two applications of the elicitors and a persistence of the effects of the first application for at least 15 days, since, even before pathogen inoculation, 24 h before and 24 h after the second application of the elicitors, there was a significant difference (t test, $p \leq 0.05$) compared to the first application, with greater PAL activity and a higher total phenol content. The effects of the elicitor can last for several days, and pre-harvest treatment using ASM reduces disease incidence and severity in some fruits post-harvest. Siddiqui (2015) reports that an application of ASM 30 days before potato harvest was effective in the control of post-harvest rot caused by *Fusarium*. ASM + strobilurin pre-harvest treatment of passion fruit reduced disease incidence and severity of fruit scab caused by *Cladosporium oxysporum* (Wilingham et al. 2002). In addition, ASM spread in pre-harvest reduced disease incidence and severity in melon fruits (Huang et al. 2000).

It needs to be highlighted that the application of the elicitors should be performed in the initial phases of the culture, to enable the preconditioning of the plants,

before the occurrence of the infection. Capdeville et al. (2003) observed a greater reduction of *Penicillium expansum* incidence and severity in apples when the harpin treatment was made in the apple trees pre-harvest, when compared with post-harvest treatment of the fruits.

PAL is the key enzyme for the production of phenolic compounds and their activity has been related to the control of various plant diseases (Durrant and Dong 2004). However, other enzymes related to plant defence, not quantified in this study, may also have been elicited in the strawberries, cv. 'Camarosa', and could have contributed to the control of the diseases and pests. Landi et al. (2014) found that in the strawberry the application of the elicitors ASM increased the activity of the enzymes phenylalanine ammonia lyase, β-1,3-glucanase, chitinase, and guaiacol peroxidase. In turn, Alamino et al. (2013) found that the post-harvest application of harpin and ASM in apples increased peroxidase activity.

The expression of defense genes in strawberry fruit treated with elicitors: chitosan, BTH, and COA, at 0.5, 6, 24, and 48 h post-treatment was analyzed. The genes were up-regulated differentially, according to the elicitor. Chitosan and COA treatments promoted the expression of key phenylpropanoid pathway genes, for synthesis of lignin and flavonoids; only those associated with flavonoid metabolism were up-regulated by BTH. The calcium-dependent protein kinase, endo-β 1,4-glucanase, ascorbate peroxidase, and glutathione-S-transferase genes were up-regulated by BTH. The K+ channel, polygalacturonase, polygalacturonase-inhibiting protein, and β-1,3-glucanase, increased in response to all tested elicitors. The enzyme activities of phenylalanine ammonia lyase, β-1,3-glucanase, chitinase, and guaiacol peroxidase supported the gene expression results. Similarity of gene expression was >72 % between chitosan and COA treatments, while BTH showed lower similarity (38 %) with the other elicitors. This study suggests the relationship between the composition of the elicitors and a specific pattern of induced defense genes.

In conclusion, this study showed that the application of ASM and harpin protein prompted the induction of resistance in strawberries, cv. 'Camarosa', by increasing the activity of the enzyme PAL, the production of phenolic compounds and photosynthesis (harpin). These effects protected the strawberries against the fungal causes of leaf blight and gray mould, and additional protection against mites was induced by the harpin protein. These results reinforce the potential that exists for the use of ASM and harpin, in association with other activities, for the integrated management of diseases and pests of strawberry, since reducing the potential of the inoculum caused by the elicitors would reduce the use of fungicides during the growing cycle. It should be highlighted that both products, harpin and ASM, are considered to have a low environmental impact and do not present a health risk to humans or animals. To date, there are no products based on harpin or ASM released for commercial use in Brazil for the control of strawberry diseases and pests (Anvisa 2015a). Finally, we agree with Walters et al. (2013) when they say that, in crop protection, as in life, one size does not fit all. If induced resistance is to be used in disease management then tailored programmes that use appropriate elicitors at the right time, at the right frequency, to responsive crop cultivars, alongside other control methods, need to be devised.

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