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Scientific Paper

Salycilic acid application in postharvest cabbage leaves butter

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Abstract

Considering the scarcity of studies related to the postharvest of kales and the inexistence of evaluation with resistance inducers in the species, we aimed to assess the effect of salicylic acid on the postharvest of collard greens over the physiochemical and biochemical variables. The experiment was conducted at the Federal Technological University of Paraná – Dois Vizinhos Campus. We used five treatments with entirely randomized design. The treatments consisted of using solutions with four concentrations of salicylic acid (0.5; 1.0; 1.5; 2.0 mM) and the control (distilled water), with four replicates of eight leaves. After the harvest, selection and standardization of the samples, the bases of the collard greens leaves were immersed in solutions with different treatments, during 10 minutes. The leaves were maintained in a B.O.D. incubator., during 192 hours, at the temperature of 8°C. The variables used for assessing the experiment were the mass loss of fresh matter, rottenness, level of vitamin C, chlorophyll, total phenols, total proteins and activity of the enzymes peroxidase and phenylalanine ammonia lyase (FAL). The application of AS maintained the contents of protein and total phenols at higher levels, as well as interfered on the activities of the peroxidase and FAL. The treatments did not present effect over the mass loss of fresh matter, and the content of vitamin C, chlorophyll and rottenness.

Keywords : *Brassica oleracea*; greenery; senescence.

Aplicacion de ácido salicílico en post-cosecha de hojas de la col forrajera

Resumen

Teniendo en cuenta la escasez de artículos relacionados con la col forrajera y la inexistencia de evaluaciones con inductores de resistencia en la especie, el objetivo del presente trabajo fue evaluar el efecto del ácido salicílico en la poscosecha de la col forrajera en las variables fisicoquímicas y bioquímicas. El experimento se llevó a cabo en la Universidad Tecnológica Federal do Paraná - Campus Dois Vizinhos. Fueran utilizados cinco tratamientos en un diseño completamente al azar. Los tratamientos constaran de lo uso de solución con cuatro concentraciones de ácido salicílico (0,5, 1,0, 1,5, y 2,0 mM) y el control (agua destilada), en cuatro repeticiones de ocho hojas. Después de la cosecha, selección y la normalización de las muestras, se sumergieron las bases de las hojas de col rizada en soluciones con diferentes tratamientos durante diez minutos. Las hojas se mantuvieron en una incubadora BOD durante 192 horas a una temperatura de 8 °C. Las variables utilizadas para la evaluación del experimento fueran la pérdida de masa de materia fresca, podredumbres, contenido de vitamina C, clorofila, fenoles totales, proteína total y la actividad de las enzimas oxidativas y fenilalanina amonio liasa (FAL). El uso de AS mantuve el contenido de proteínas y fenoles totales en niveles más altos e interfirió en la actividad de las peroxidasas y FAL. Los tratamientos no tuvieron efecto sobre la pérdida de masa de la materia fresca, o contenido de vitamina C, clorofila y podredumbres.

Palabras clave: *Brassica oleracea*; hojas; hortalizas; senescencia.

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Aplicação de ácido salicílico na pós-colheita de folhas de couve manteiga

Resumo

Dada a escassez de itens relacionados à couve e falta de avaliações com indutores de resistência na espécie, o objetivo deste estudo foi avaliar o efeito do ácido salicílico na pós-colheita de couve nas variáveis físico-químicas e bioquímicas . O experimento foi conduzido na Universidade Tecnológica Federal do Paraná - Campus Dois Vizinhos. Foram usados cinco tratamentos delineamento experimental inteiramente casualizado. Os tratamentos consistem na utilização da solução com quatro concentrações de ácido salicílico (0,5, 1,0, 1,5, e 2,0 mM) e de controlo (água destilada) em quatro réplicas de oito folhas. Após a colheita, selecção e normalização das amostras, as bases da couve em soluções com diferentes tratamentos foram imersas durante dez minutos. As folhas foram mantidas numa incubadora de BOD para 192 horas a uma temperatura de 8 °C. As variáveis utilizadas para a avaliação do experimento foram a perda de massa da matéria fresca, apodrecendo, a vitamina C, clorofila, fenóis totais, proteína total e atividade de enzimas oxidativas e fenilalanina amônia liase (FAL). A utilização de AS mantido o teor de proteínas totais e fenóis em níveis mais elevados e interferiu com a actividade de FAL e peroxidases. Os tratamentos não tiveram efeito sobre a perda de massa da matéria fresca, ou vitamina C, clorofila e podridão.

Palavras chave: *Brassica oleracea*; folhas; legumes; senescência.

Introduction

The collard green (*Brassica oleracea*) belong to the Brassicaceas Family; it is a herbaceous upright plant with sub-woody stem. It emits leaves continuously (VIEIRA, 2006), distributed around the stem, in form of rosette and presents a well developed rounded ,limb, with long petiole and well detached nerves. In Brazil, the collard green rarely produces inflorescence, presents certain tolerance to heat, remaining productive during several months (BEZERRA et al., 2005). It is a greenery whose leaf is the edible part, being rich in vitamin A and C and well appreciated in culinary, preferable when freshly picked, green and without signs of dehydration or rottenness (FILGUEIRA, 2003).

Due to the data presented, the production of food considered healthy, free of pesticides and chemical products, has been more sought by consumers over the past few years, which makes the resistance inducers an alternative with potential for using it in foods. According to STADNIK (2000) and HAMMERSCHIMIDT et al., (2001), the induction of resistance is conceptualized as an activation of a resistance state against diseases, which is induced systematically in plants through the use of external biotic or abiotic agents, without any alteration of the plant's genome, occurring in a non-specific way, through the activation of genes that codify for diverse defense responses.

The first study that had detailed analysis about the resistance induction was by ROSS (1961), who demonstrated through a laboratory experiment that tobacco plants necrosed by the inoculation of the inferior leaves with the TMV (tobacco mosaic virus), developed systemic resistance against several pathogens, resulting in the conception of the term "acquired systemic resistance" (RSA) (SILVA, 2008).

The genes involved in the RSA are associated, mainly, with the accumulation of salicylic acid (AS), which is an indicator for the expression of RP proteins (GRÜNER et al., 2003; JALALI et al., 2006). According to YALPANI et al., (1991), the biosynthetic pathway of the salicylic acid is apparently initiated with the conversion of phenylalanine to transcinnamic acid, catalyzed by the enzyme phenylalanine ammonia lyase (FAL). From this conversion, the transcinnamic acid seems, then, to follow two ways. In one of them, there is a formation of benzoic acid that, after the action of the enzyme benzoic acid 2-hydroxylase, converts in AS. The other, the transcinnamic acid is hydroxylated to acid 2-coumaric, which is, then oxidized to AS (RYALS et al. 1994; STRACK, 1997). However, studies show as the main pathway of formation of the AS is that where the intermediary is the benzoic acid (VERNOOIJ et al., 1994).

The main physiological role attributed to the AS in the plant is to function as an indicator molecule, inducing to express resistance against the attack of predators. This function was suggested as a result of the AS to accumulate in plants subjected to adverse conditions, whether by pathogen attack

or by the treatment with chemical products, and also by the AS property of inducing the expression of genes linked to several RP proteins (MARTINEZ et al., 2000). Exogenous applications of AS, inducing effectively the gene expression of proteins involved on the induction of systemic resistance, led to the investigation of the role of the endogenous AS in the resistance to diseases (CHET, 1993). According to this hypothesis, the AS can be a messenger that activates the resistance against pathogens, including the synthesis of RP proteins (CAMPOS, 2009).

Furthermore, the AS is a natural phenolic compound that can decrease the ethylene synthesis in the plants, slowing the effects of this hormone, because it can reduce the activity of the ACC oxidase, precursor enzyme for its synthesis (ALTVORST and BOVY, 1995). This is an important factor, mainly for plants that are too perishable, such as fruits, greenery and flowers. Among the plants with low useful life after-harvest we can highlight the blackberry, acerola, collard green, spinach and roses.

Due to the fact that the collard green leaves have elevated perishability, and consequently a short period after-harvest, it becomes necessary the to search to products that are able to provide an increase of after-harvest life of this species, whereas the resistance inducers are related to the activation of defense mechanisms of the vegetal, and can act over the quality preservation after-harvest.

Therefore, the salicylic acid can be an alternative product for the quality maintenance after-harvest of collard green leaves. Due to its antagonistic action to ethylene, this can prolong the life after-harvest, by inqilifing inhibition inquilifing deleterious effects of this hormone (IMRAN, 2007). Besides, the salicylic acid when applied exogenously is capable of inducing the production of proteins related to pathogenesis (RP proteins), protecting the plants from pathogen attacks (SPLETZER and ENYEDI, 1999).

Considering the scarcity of studies related to the post-harvest of collard greens and the inexistence of assessment of resistance inductors in this species, we aimed with this study to evaluate the effect of salicylic acid on the post-harvest conservation of collard green leaves.

Material and Methods

The experiment was conducted in the Laboratory of Phytopathology at the Federal Technological University of Paraná – Dois Vizinhos Campus, Paraná. The collard green leaves were harvested a garden with agroecological cultivation system in the city of Dois Vizinhos. Subsequently, the leaves were transported to the Laboratory of Phytopathology for selection, standardization and weighing of the samples. After, we applied in the leaves the treatment with salicylic acid (AS), differing by the concentration used in the solution, being 0.5; 1.0; 1.5 and 2.0 mM and the control treatment (distilled water). The basis portion of the leaves were subjected to the treatments by immersion, for 10 minutes, followed by its conditioning in transparent polyethylene bags (15 microns) with the upper part open, and storage in B.O.D. at a temperature of 8ºC. We used the entirely randomized experimental design with four replicates of 8 leaves per parcel.

After 192 hours of storage, we assessed the loss of fresh matter mass, the chlorophyll level, vitamin C and rottenness of the leaves. During the experiment, in intervals of 24, 48, 96 and 192 hours after the application of the treatments with AS, we also took samples of the leaves in order to conduct biochemical analysis of proteins, total phenols and activity of the enzymes phenylalanine ammonia-lyase and peroxidase.

The loss of fresh matter mass was obtained by the difference of fresh matter of the samples in the day of the experiment installation and the value found in the weighing at the end of the experiment, expressed in percentage. The evaluation of rottenness was conducted visually, observing the characteristic symptoms of pathogen attacks. For the determination of chlorophyll level we used a chlorophyllmeter ClorofiLOG® model CFL 1030, determining "a", "b" and total. The level o vitamin C was assessed by the method of Tillmans (INSTITUTO ADOLFO LUTZ, 1985).

For the protein dosage we employed the test of BRADFORD (1976), where we removed a sample of the leaves of each experimental unit, placed it in a porcelain pot and macerated it with buffer solution of Phosphate 0.2 Molar pH 7.5, obtaining, thus, the extract. This extract was placed in eppendorfs properly identified, taken to the centrifuge, where they remained for 10 minutes at 12.000 rpm and at 4ºC. After being removed from the centrifuge, we transferred 40 micro liters of the supernatant extract to test tubes, being added more 460 micro liters of distilled water and 1.0 mL of the reagent Bio-Rad. The solutions were stirred and, conditioned in cuvettes

in order to conduct the reading at 630 nanometers in spectrophotometer, obtaining, thus, the value of absorbance.

The determination of activity of the phenylalanine ammonia-lyase (FAL) was conducted by colorimetric quantification of the transcinnamic acid released from the substrate phenylalanine, according to the methodology described by KUHN (2007), where we used a sample of the leaves of each experimental unit, placed it in a porcelain pot and macerated with more 3.0 mL of buffer TRIS – HCl pH 8.0. This extract was conditioned in eppendorf tubes, properly marked and taken to the centrifuge for 10 minutes, at 4ºC and 6000 rpm. After, we transferred an aliquot of 200 µL to an identified test tube, adding more 3.0 mL of extraction buffer. The solution was stirred, obtaining, thus, the enzymatic extract. Of this extract, 1.5 mL was transferred to another test tube, with more 1.0 mL of extraction buffer and 0.5 mL of phenylalanine. Again, this solution was stirred for the homogenization. And after, the tubes were incubated in Bain Marie for 45 minutes at 40ºC. After removing from the Bain Marie, the tubes were placed in ice bath for 5 minutes in order to interrupt the reaction and thus be able to conduct the reading through the spectrophotometer at 290 nm.

For the quantification of total phenols we used the method adopted by BIELESKI and TURNER (1966) and JENNINGS (1991), removing a sample of the leaves from each experimental unit, placing it in a porcelain pot and macerating it with more 3.0 mL of the MCA solution (methanol, chloroform and water (6.0/2.5/1.5). This extract was conditioned in eppendorf tubes properly identified, which were taken to the centrifuge for 20 minutes, at 20ºC and 6000 rpm. After the centrifugation, we removed 2.0 mL of the supernatant and placed it in identified test tubes, along with 1.0 mL of chloroform and 1.5 mL of distilled water, stirring it right after. Following, we removed 0.5 mL of the upper part of the supernatant, placed in another test tube along with 0,5 mL of distilled water and 0.5 mL of the reagent Folin – Cocalteau diluted 1:10. After 15 minutes, we added 5.0 mL of the alkaline reagent composed by sodium carbonate at 2% in solution of sodium hydroxide 0.1 N. Again this solution was stirred and after 50 minutes we conducted the reading through the spectrophotometer at 760 nm in order to obtain the values of absorbance.

The extraction and determination of the enzymatic activity of peroxidase were conducted through the method recommended by MATSUNO and URITANI (1972), where we took a sample of the leaves from each experimental unit, placed it in a refrigerated porcelain pot, because this analysis must be conducted at temperatures under 4ºC. The samples were macerated with 3.0 mL of buffer phosphate 0.05 M (pH 7) with more 0.005g of polyvinylpyrrolidone. The extract was conditioned in eppendorf tubes properly identified, which were taken to the centrifuge for 20 minutes at 4ºC and 5000 rpm. After the centrifugation, we removed 2.0 mL of the supernatant and placed it in identified test tubes, where there was a solution of 3.0 mL of buffer citrate (pH 5.0) plus 0.5 mL of hydrogen peroxide at 3% and more 0.5 mL of guaiacol 0.5%. The solution was stirred and placed for 15 minutes under Bain Marie at 30ºC and, after, 10 minutes in ice in order to stop the reactions. Finally, we added 0.5 mL of sodium bisulfite, stirred and made the reading at 450 nm through the spectrophotometer, obtaining thus the values of absorbance.

The data were subjected to variance analysis ($p \leq 0.05$), presented using descriptive statistics (average ± standard deviation) and regression analysis, using the program ASSISTAT (SILVA and AZEVEDO, 2009).

Results and Discussion

The results obtained in the present experiment demonstrate that the application of salicylic acid did not interfere in the reduction of fresh matter mass loss, in levels of vitamin C, chlorophyll and on incidence of rottenness. The loss of fresh matter mass is caused mainly by water loss in the leaves, through processes of transpiration and respiration. Even though there was no significant effect of the treatments over the loss of fresh matter mass, it occurred within the patterns of normality, whose average value was of 4.681%. The loss of fresh matter mass in greenery can also be observed by the aspect of wilting, which was not observed in any of the treatments. Similarly, WEBER et al. (2012) did not find difference in loss of fresh matter mass in fruits of passion fruit trees treated with salicylic acid. The vitamin C level in the leaves of collard green varied from 96 to 132 mg 100 g^{-1} of tissue, value considered within the observed and cited in the literature (NEPA/UNICAMP, 2006).

As for the level of chlorophylls, there was no difference for the application of AS; the average value for chlorophyll a was 36.88 mg g^{-1}, chlorophyll

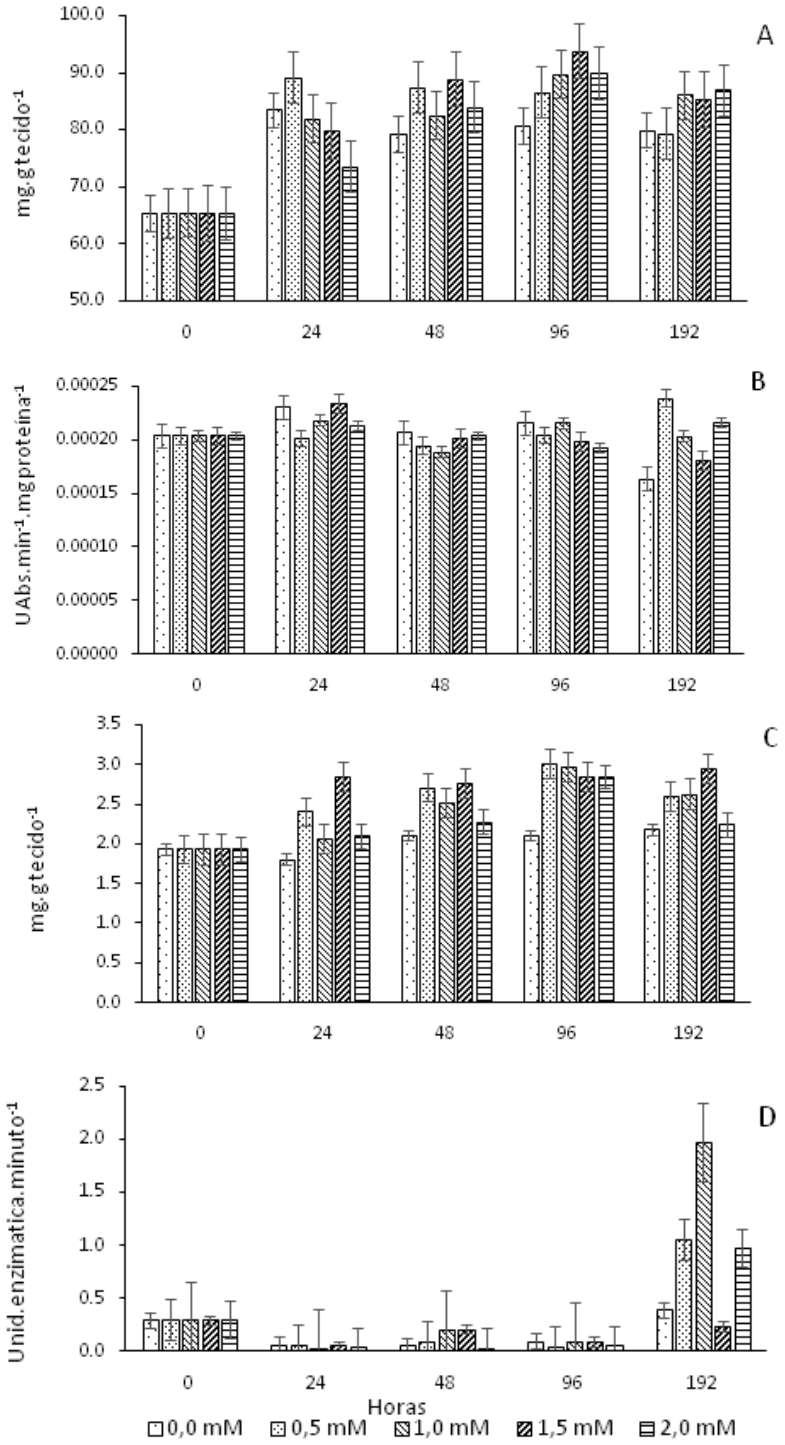

*Figure edited in portuguese by the author.

Figure 1. Protein level (A), activity of the enzyme FAL (B), phenol lelvel (C) and peroxidase (D) of collard green leaves, assessed at 0, 24, 48, 96 e 192 hours after the application of five concentrations of salicylic acid. Vertical bars indicate the standard deviation. UTFPR. Dois Vizinhos – PR, 2013.

b 6.14 mg g^{-1} and total chlorophyll 43.02 mg g^{-1}. The chlorophyll is an important parameter, because the levels of chlorophyll a and b are directly related to the green coloration. In case the treatments had interfered on the degradation of this pigment, it would have occurred a predominance of yellow, which is not a desirable characteristic for the commercialization of collard greens. We did not observe rottenness symptoms in this study, which can be related to the management given during the cultivation and the storage conditions.

The use of AS interfered over the total protein levels in all treatments (Figure 1A), observed 24 hours after the application. This is possibly due to the higher metabolic activity occurred by the stress of detaching the leaves from the mother plant. After this period, we observed that in the control treatments and in the lowest concentration (0.0 e 0.5 mM) there was a stabilization or decrease of protein levels. As for the other concentrations, the opposite occurred, that is, an increase in protein levels, reaching its maximum level 96 hours after the AS application. These changes are possibly related to the synthesis of RP proteins, such as the chitinases and β-1.3-glucanases. The exogenous application of salicylic acid on several different plants induces the expression of the genes of RP proteins, not only in the local application, but also in a systemic way, suggesting that the AS acts as an indicator of acquired systemic resistance (SAR), which is a form of induced resistance in order to increase the plant's defense against subsequent infections and pathogen attacks (KESSMANN et al., 1994). However, after 96 hours we can observe that for all AS treatments, the protein levels decreased. We suppose that this is linked to the fact that the collard green leaves were in the process of senescence, and, thus, were in the natural condition of protein degradation.

In Figure 1B it is possible to verify that the activity of the FAL enzyme presented an oscillatory behavior during the experiment in all AS solutions used. However, there was no significant difference between them. This is possibly due to the fact that the activation of the FAL occurred in different moments of the assessments, that is, in intervals that were not observed in this experiment, since in the moment of the assessments they were already in similar levels to the control treatment. Thereby, we suggest further studies that contemplate the evaluation of the FAL enzyme activity throughout the experiment, with intervals of 12 and 24 hours, as well as the insertion of analysis involving the enzymes chitinases and β-1,3-glucanases.

Although we have not found the FAL activity, the AS treatments induced the production of phenolic compounds, which can be observed in the assessments of total phenols (Figure 1C). In all assessments and for all the studied concentrations, the total phenols maintained greater levels in relation to control. This demonstrates that the pathway to phenolic compounds was activated by the treatments, being observed an increase after 24 hours and with higher prominence in the evaluation conducted 72 hours after the application of the inducers.

NUNES et al. (2008) found similar results working with different concentrations of AS in the cultivation of medicinal plant Hypericum polyanthemum, where the results demonstrated that the plants treated with AS significantly increased the production of phenolic compounds on the different analyzed parts.

Similarly to the FAL, the activity of peroxidases had few changes 96 hours after the AS application, without the occurrence of statistical difference among the treatments (Figure 1D). After 96 hours, great activities occurred in all treatments, mainly with the concentrations of 0.5; 1.0 e 2.0 mM. We suppose that from this moment, the leaves are beginning their senescence process, which reinforces the hypothesis raised previously regarding proteins, in which they are in the process of degradation because of the senescence and, with that, a consequent elevation of peroxidases occur, which are linked to cellular damages. Such results strengthen the need for further studies in relation to the activity of the FAL and peroxidases, since these enzymes act on the main pathways of phenol formation, which were stimulated by the treatments with salicylic acid.

Conclusions

The application of AS maintains the levels of proteins and total phenols at more elevated levels, as well as interferes on the activity of the enzymes peroxidase and FAL. The treatments did not present effect over the loss of fresh matter mass, vitamin C content, chlorophylls and rottenness.

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