ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Calcium content on apple fruit influences the severity of *Penicillium expansum*

El contenido de calcio en fruta de manzana influencia la severidad de *Penicillium expansum*

Guerrero-Prieto VM[1], DI Berlanga-Reyes[2], JL Jacobo-Cuellar[1], C Guigón-Lopez[3], DL Ojeda-Barrios[4], GD Ávila-Quezada[4], A Núñez-Barrios[4], OA Hernández-Rodríguez[4]

Abstract. Calcium content and damage severity of *Penicillium expansum* because of its high concentration on "Red Delicious" postharvest apples were evaluated during the 2012-2013 winter in Cuauhtemoc, Chih. Mexico. Fruit weight, diameter, total soluble solids, pulp firmness and starch index were also determined. *Penicillium expansum* was inoculated into two wounds of eight mm diameter and 10 mm depth, on a total of 20 apple fruits per treatment. The inoculum was 0.2 mL of a suspension containing 1×10^8 conidia/mL. After inoculation, apple fruit was stored at 0 °C and 90% relative humidity during five weeks. Treatments were: apple fruit with high (2.28 mg/100 g fresh weight) and low (2.16 mg/100 g fresh weight) calcium content, with and without *P. expansum*, and a control which was inoculated with sterile water. The greater the fruit Ca content, the lower the severity of the fruit area affected by *P. expansum* (i.e., 27 cm2 affected with high Ca content vs. 34 cm^2 with low Ca content).

Keywords: Calcium; Cold Storage; Nutrients; Postharvest Diseases.

Resumen. El contenido de calcio y la severidad provocada por una alta concentración de *Penicillium expansum* en manzana "Red Delicious" en poscosecha fue evaluado en el invierno 2012-2013 en Cuauhtémoc, Chih. México. Peso, diámetro, sólidos solubles totales, firmeza e índice de almidón en la fruta también fueron evaluados. *Penicillium expansum* se inoculó en dos pozos de ocho mm de diámetro por 10 mm de profundidad, en un total de 20 manzanas por tratamiento. El inóculo fue de 200 µL de una suspensión de 1×10^8 conidios/mL del patógeno. Después de la inoculación, la manzana se refrigeró a 0 °C y 90% de humedad relativa durante cinco semanas. Los tratamientos fueron; fruta con alto (2,28 mg/100 g de peso fresco) y bajo (2,16 mg/100 g de peso fresco) contenido de calcio, con y sin *P. expansum* y un testigo inoculado con agua estéril. La severidad del área afectada por la presencia de *P. expansum* se redujo cuanto mayor fue el contenido de Ca de la fruta (27 cm^2 de área afectada con alto contenido de Ca vs 34 cm^2 con bajo contenido de Ca.)

Palabras clave: Enfermedades poscosecha; Nutrientes; Daño en Fruta; Refrigeración.

[1] Facultad de Ciencias Agrotecnológicas, Universidad Autónoma de Chihuahua, Ext. Cuauhtémoc, Chih. Av. Presa de La Amistad # 2015, Cuauhtémoc, Chih. C.P. 31510, México.
[2] Centro de Investigación en Alimentación y Desarrollo, A.C., Unidad Cuauhtémoc, Av. Río Conchos s/n, Parque Industrial, Apdo. Postal 781, Cuauhtémoc, Chih, C.P. 31570, México.
[3] Centro de Investigación en Recursos Naturales. DGETA-SEP. Salaices, Chih. México.
[4] Facultad de Ciencias Agrotecnológicas, Universidad Autónoma de Chihuahua, Campus I, Chihuahua, Chih. C.P. 31500, México.
Address correspondence to: Dr. VM Guerrero-Prieto. Facultad de Ciencias Agrotecnológicas, Universidad Autónoma de Chihuahua, Ext. Cuauhtémoc, Chih. Av. Presa de La Amistad # 2015, Cuauhtémoc, Chih. C. P. 31510. México, *e-mail:* vguerrero@uach.mx
Received 13.I.2015. Accepted 25.VIII.2016.

INTRODUCTION

Several *Penicillium* species are among the fungi which more frequently damage postharvest apple fruit (Vico et al., 2010). *Penicillium expansum* (Link) Thom and *P. solitum* Westling are the two species more frequently reported both in the fruit and in cold storage rooms (Amiri & Bompeix, 2005; Vico et al., 2010; Yu et al., 2016).

In Chihuahua, Mexico, both species have recently been reported as the most frequent ones on postharvest apple fruit (González-Martínez, 2014). *Penicillium expansum* is responsible for economic losses in the United States of up to 4.4 million dollars per year (Vico et al., 2010). To control *P. expansum*, chemical synthetic fungicides are commonly used by the growers (Guerrero-Prieto et al., 2004). Also, the development of resistance of *P. expansum* to the common fungicides has been reported (Mari et al., 2014).

Nonetheless, consumers and health authorities, are getting increasingly concerned with the utilization of synthetic fungicides due to their residues on foods and the environment, which make their use questionable (Janisiewickz & Korsten, 2002; Muñoz et al., 2011). This is because consumers and health authorities demand safe foods. An alternative method to control *P. expansum* is the application of calcium on the fruit (Conway et al., 1991). Calcium postharvest sprays have been used to increase calcium content in the cell wall, since calcium is effective on delaying fruit senescence, giving more firm fruits. These are of higher quality, and less susceptible to postharvest diseases (Chardonnet et al., 2003).

Many techniques to supply calcium in the fruit tissue cell wall have been developed. These techniques include heat treatments, fruit immersion, vacuum infiltration, pressure infiltration, addition of surfactants and/or protection agents, or a combination of these techniques (Chardonnet et al., 2003). Moor et al. (2006) reported that in many regions of the world, apple growers use, on a routine basis, calcium sprays to reduce fruit damage and/or postharvest apple physiological disorders (Park et al., 2006). Conway et al. (2002) mentioned that an adequate field management to provide calcium to the fruit, plus direct applications to it on a pre- and postharvest basis, are the best way to supply this nutrient on adequate amounts.

Calcium is part of the cell wall structure, which contributes to fruit firmness (Tobias et al., 1993; Roy et al., 1994; Chardonnet et al. 2003; Hepler, 2005; Hepler & Whinship, 2010). The cell wall is also a natural barrier against phytopathogenic fungi (Vico et al., 2010). Calcium ion (Ca^{2+}), cross-linked in the cell wall, is involved in maintaining cell wall integrity. This is when it combines to carboxyl groups of the polygalacturonate chains, which are found mainly in the middle lamella and in the primary cell wall (Tobias et al., 1993; Chardonnet et al., 2003). The cell wall contains pectin, cellulose, hemicellulose and proteins. Pectin is present in combination with hemicellulose, and provides strength to the cell wall, tissue organization and determines fruit and vegetable texture (Vico et al., 2010).

Calcium plays a primary role to determine cold storage capacity in fruits. This mineral element also affects fruit senescence when altering intra and extracellular processes (Tobias et al., 1993; Conway et al., 2002; Buccheri & Di Vaio, 2004).

Finally, calcium is essential to defend apple fruit against postharvest pathogens. The objective of this work was: to evaluate the response of cold-stored apple fruit, cv Red Delicious, to either low or high calcium levels when exposed to *Penicillium expansum.*

MATERIALS AND METHODS

Microorganisms. *Penicillium expansum* Link Thom strain was obtained from the "Microorganismos de la Zona Templada" ("Temperate Zone Microorganisms), at the Center for Research in Food and Development (CIAD, A. C., www.ciad.mx), Cuauhtémoc Unit, Cuauhtemoc, Chih. Mexico.

Apple fruit in the field. Apple fruits from different apple orchards were harvested and cold stored. These fruits had a different number of preharvest applications with calcium chloride in the field per growing cycle, according to the doses and timing used by apple growers. Apple fruits were randomly selected as follows: (1) fruits with eight or more calcium field sprays, where calcium content was 2.28 mg/100 g fresh weight (calcium level one); (2) fruits with four or less calcium field sprays, where calcium content was 2.16 mg/100g fresh weight (calcium level two). These fruits [i.e., (1) and (2)] were used in the two experiments. Experiments consisted of using Red Delicious apple fruits, originally harvested from the orchards, after storing them either during three months (experiment one) or 203 days (experiment two). Storage conditions were 0 °C and 90% relative humidity (HR) in the cold storage "Arroyo Seco Company", Cuauhtémoc, Chih. México.

Variables quantified. Calcium content was determined on apple fruits at the Center for Research in Food and Development (CIAD), A. C. Cuauhtémoc, Chih. Mexico Unit. Experiments were run twice. Other variables, such as weight (g, Ohaus digital balance); equatorial diameter (mm, Cranston caliper) and rot area (cm^2); total soluble solids (° Brix, Atago, digital refractometer); pulp firmness (N and lbs/in^2, TA-XT2i Universal texturometer) and Iodine-starch index (1-6 scale), were also determined on apple fruits.

Treatments. Four treatments were defined: (1) ACaCP = apple fruit calcium level one, inoculated with *P. expansum*; (2) ACaSP = apple fruit with calcium level one, without *P. expansum* inoculation; (3) BCaCP = apple fruit with calcium level two, *P. expansum* inoculated, and (4) BCaSP = apple fruit with calcium level two, without *P. expansum* inoculation. Treatments were assigned randomly.

Experiments, replications and treatment application. Each treatment was applied on 20 apple fruits, and each apple fruit had two wounds (eight mm diameter and 10 mm depth). Forty replications per treatment were included in the experiments. Each wound was inoculated with 200 µL of a suspension containing 1×10^8 conidia/mL of *P. expansum*. Control treatments consisted on sterile water inoculation on each wound of apple fruit, corresponding to both calcium levels. Two experiments (1 and 2) were run in the way previously described. Fungus inoculum suspensions were prepared by using a hemacytometer from a culture of 12 days grown at 26 ˚C.

Cold storage of the treatments. Once all treatments were established, apple fruits were stored during a five-week-storage period at 0 ˚C and 90% RH.

Severity evaluation. Increases in apple fruit lesion diameter and area (cm^2) due to *P. expansum* decay were weekly evaluated during one month, on each inoculated wound, on each treatment and replication, and on each of the two experiments.

Statistical analysis. A variance homogeneity test was first applied to the results for lesion diameter due to *P. expansum* severity on the apple fruits, with treatments either inoculated or not (i.e., controls), at both calcium levels. An analysis of variance was done when homogeneity was found. Statistical significant differences among treatment means were determined using the Tukey Test (α=0.05). The Statistical Analysis System (i.e., version 6.12. Cary, NC USA) was used for data analyses. When variance homogeneity was not detected, mean values were compared using the non-parametric Kruskal-Wallis test; if statistical differences were detected among treatment means, the Mann-Whitney test (Sprent & Smeeton, 2001) was used at a 95% level of significance to determine statistical differences among treatments. Lesion severity development was analyzed with non-linear models using the SAS 6.12 version (SAS, 2002). A modified Weibull non-lineal model was used using the following equation: $Y = 1-\exp(-DAI/b)^c$; where Y = lesion growth proportion; DAI = number of days after inoculation; b = inverse lesion growth rate estimator (1/b), and c = a parameter as a curve's shape function (Pennypacker et al., 1980).

RESULTS

Calcium content was statistically different between the two calcium levels. Apple fruit maturity indexes obtained for the two evaluations indicated that apple fruits used for experiment one (November 1, 2012) had a less advanced maturity stage than apple fruits used on experiment two on February 22, 2013 (Table 1).

Penicillium expansum severity was lower on apple fruits with calcium level one (with a higher Calcium content) than on those with calcium level two (with a lower Ca content) (Table 2). Lesion growth increment rates were statistically equal with respect to time, according to the model indicators generated to explain lesion development after *P. expansum* inoculation (Table 3). The results obtained in this experiment showed that *P. expansum* severity development on apple fruits was equal on fruits having either high or low calcium contents. However, final lesion was higher (34.68 cm^2) on apple fruits with low than high Ca contents.

DISCUSSION

Calcium levels, in both cases, were lower than those reported by Chardonnet et al. (2003) on Golden Delicious apple fruits during cold storage (2.3 ± 0.04 mg/g dry weight). They were also lower than those shown by Ernani et al. (2008) on

Table 1. Maturity indexes of Red Delicious apple fruits used on the experiments 2012-2013.
Tabla 1. Índices de madurez en manzana Red Delicious utilizadas en los experimentos 2012-2013.

Date of evaluation	Fruit Weight (g)	Fruit Diameter (mm)	Total Soluble Solids (˚ Brix)	Pulp Firmness		Iodine-starch (1-6)
				(N)	(Lb/inch^2)	
1-Nov-2012						
calcium 1	147.2	68.8	15.9	62.9	14.1	3.7
calcium 2	158.1	74.1	15.3	52.3	11.8	3.7
22-Feb-2013						
calcium 1	125.2	69.1	16.2	32.4	7.3	6
calcium 2	131.3	73.8	17.8	31.9	7.2	6

Table 2. Decay severity (cm^2) due to *Penicillium expansum*, on Red Delicious apple fruits with different calcium contents (2012-2013).
Tabla 2. Severidad de podredumbre (cm^2) por *Penicillium expansum* en manzanas Red Delicious causada por diferentes contenidos de calcio (2012-2013).

Treatment	Experiment 1	Experiment 2	Both experiments integrated
BCaCP	28.2 a	43.0 a	34.68 a
BCaSP	2.0 c	2.5 c	2.34 c
ACaCP	23.5 b	30.4 b	27.33 b
ACaSP	1.8 c	2.1 d	2.01 d

Medians with the same letter within columns are statistically equal (P≤0.05) according to the Mann-Whitney test.
BCaCP = Fruit with low calcium content and inoculated with *P. expansum.*
BCaSP = Fruit with low calcium content without inoculation with *P. expansum.*
ACaCP = Fruit with high calcium content inoculated with *P. expansum.*
ACaSP = Fruit with high calcium content without inoculation with *P. expansum.*

Table 3. Weibull modified model indicators for lesion increment (cm^2) due to *Penicillium expansum* severity after inoculation of apple fruits with low and high calcium contents.
Tabla 3. Indicadores del Modelo de Weibull modificado respecto al incremento de lesión (cm^2) causado por *Penicillium expansum* después de la inoculación de las manzanas con bajo y alto contenido de calcio.

Treatment	Model $y = 1 - e^{[-(t/b)^c]}$	Slope value (1/b)	(c) shape parameter model value	R^2 (%)	CV (%)
BCaPe	$y = 1-e^{[-(t/28.9297)^{5.1029}]}$	0.03456655 a	5.1029	98.3	17.83
ACaPe	$y = 1-e^{[-(t/32.8109)^{4.7958}]}$	0.03047768 a	4.7958	99.0	11.67

Slope values with the same letter are statistically equal at a higher than 95% confidence level.
BCaPe= Low calcium (level two) content on apple fruits inoculated with *P. expansum.*
ACaPe= High calcium (level one) content on apple fruits inoculated with *P. expansum.*

Gala apple fruits (4.4 mg/100g). Despite the calcium levels in the fruits used in our experiment were lower than those reported in other studies, there were statistically significant differences between the two study treatments and on fruit decay by *P. expansum* (Table 2).

Differences in maturity indexes (Table 1) might be because the fruits on 22 February 2013 had 113 more days of cold storage than fruits used on November 2012. Calcium plays a primary role determining the duration of cold storage on fruits. Calcium also affects fruit firmness and senescence by altering intra- and extra-cellular processes (Tobias et al., 1993; Conway et al., 2002; Buccheri & Di Vaio, 2004; Hepler & Winship, 2010). Tobias et al. (1993) stated that 60% of the cell calcium is concentrated in the cell wall and middle lamella. This explains why there was a response to the calcium content on both treatments used in the study (Table 2), even though both calcium levels were lower than those reported by other researchers. Wojcik (2001) reported that "Jonagold" apple fruits were more resistant to fruit decay than control fruits after they were sprayed with high doses of calcium during summer and fall.

The higher severity infection on apple fruits with calcium level two (Table 2) might be because of the fruit conditions which favored infection development. These conditions might include the dissolution of the middle lamella and cell wall on the inoculated apple fruits, as it has been reported by Chardonnet et al. (2003). These authors observed an increment on the susceptibility of apple fruits to fungal pathogens, associated to cell wall degradation and carbohydrate metabolism in the cell wall (Tables 2 and 3).

CONCLUSIONS

High calcium contents on the apple fruits used in this experiment had a direct effect on the reduction of the severity infection to *Penicillium expansum.*

ACKNOWLEDGEMENTS

Authors thank Mr. Emilio Rentería Torres, "Arroyo Seco Company" cold storage facility owner, and Mr. Gabriel Amado Baeza Lopez, General Manager of the same cold storage facility, for their constant support to the present and other research projects.

REFERENCES

Amiri, A. & G. Bompeix (2005). Diversity and population dynamics of *Penicillium* spp. on apples in pre- and postharvest environments: consequences for decay development. *Plant Pathology* 54: 74-81.

Buccheri, M. & C. Di Vaio (2004). Relationship among seed number, quality, and calcium content in apple fruits. *Journal of Plant Nutrition* 27: 1735-1746.

Chardonnet, C., O. Craig, S. Charron, C.E. Sams & W.S. Conway (2003). Chemical changes in the cortical tissue and cell walls of calcium-infiltrated 'Golden Delicious' apples during storage. *Postharvest Biology and Technology* 28: 97-111.

Conway, W.S., C.E. Sams, J.A. Abbott & B.D. Bruton (1991). Postharvest calcium treatment on apple fruit to provide broad-spectrum protection against postharvest pathogens. *Plant Disease* 75: 620-622.

Conway, W.S., C.E. Sams & K.D. Hickey (2002). Pre- and postharvest calcium treatment of apple fruit and its effect on quality. *Acta Horticulturae* (ISHS) 594: 413-419.

Ernani, P.R., C. Jaques-Dias, V. Talamini do amarante, D. Cardoso-Ribeiro & D.A. Rogeri (2008). Preharvest calcium sprays were not always needed to improve fruit quality of 'Gala' apples in Brazil. *Revista Brasileira de Fruticultura* 30: 892-896.

González-Martínez, N.I. (2014). Microorganismos epifitos presentes en manzana "Golden Delicious" en poscosecha. MS Thesis. FACIATEC. Autonomus University of Chihuahua. Mexico. 100 p.

Guerrero-Prieto, V., G. Trevizo-Enriquez, A. Gardea-Béjar, C. Figueroa-Valenzuela, A. Romo-Chacón, C. Blanco-Pérez & E. Curry (2004). Identificación de Levaduras Epífitas Obtenidas de Manzana (*Malus sylvestris* Mill.) para Control Biológico Poscosecha *Revista Mexicana de Fitopatología* 22: 223-230.

Hepler, P.K. (2005). Calcium: a central regulator of plant growth and development. *Plant Cell* 17: 2142-2155.

Hepler, P.K. & L.J. Winship (2010). Calcium at the cell wall-cytoplasm interface. *Journal of Integrative Plant Biology* 52: 147-160.

Janisiewicz, W. & L. Korsten (2002). Biological control of postharvest diseases of fruits. *Annual Review of Phytopathology* 40: 411-441.

Mari, M., A. Di Francesco & P. Bertolini (2014). Control of fruit postharvest diseases: old issues and innovative approaches. *Stewart Postharvest Review* 2014: 1-1.

Moor, U., K. Karp, P. Poldma, L. Asafova & M. Starast (2006). Postharvest disorders and mineral composition of apple fruits as affected by pre-harvest calcium treatments. *Acta Agriculturae Scandinavica Section B-Soil and Plant Science* 56: 179-185.

Muñoz, A., B. López-García, A. Veyrat, L. González-Candelas & J.F. Marcos (2011). Comparative analysis of the sensitivity to distinct antimicrobials among *Penicillium* spp. causing fruit postharvest decay. *Phytopathologia Mediterranea* 50: 392-407.

Park, E., T. Solomos, J.L. McEvoy, W.S. Conway & C.E. Sams (2006). The effect of calcium on the polygalacturonase activity by *Colletotrichum acutatum* in apple fruit. *Plant Pathology Journal* 5: 183-190.

Pennypacker, S.P., H.D. Knoble, C.E. Antle & L.V. Madden (1980). A flexible model for studying plant disease progression. *Phytopathology* 70: 232-235.

Roy, S., W.S. Conway, A.E. Watada, C.E. Sams, C.D. Pooley & W.P. Wergin (1994). Distribution of the anionic sites in the cell wall of apple fruit after calcium treatment. Quantitation and visualization by a cationic colloidal gold probe. *Protoplasma* 178: 156-167.

SAS Institute (2002). SAS/STAT _User's Guide, Software version 9.0. SAS Institute Inc., SAS Campus Drive, Cary, NC 27513-2414, U.S.A.

Sprent, P. & N.C. Smeeton (2001). Applied nonparametric statistical methods. 3rd ed. Chapman and Hall CRC. Washington, D.C., USA. 461 p.

Tobias, R.B., W.S. Conway, C.E. Sams, K.C. Gross & B.D. Whitaker (1993). Cell wall composition of calcium-treated apples inoculated with *Botrytis cinerea*. *Phytochemistry* 32: 35-39.

Vico, I., W. Jurick II, M. Camp, W. Janisiewicz & W. Conway (2010). Temperature suppresses decay on apple fruit by affecting *Penicillium solitum* conidial germination, mycelial growth and polygalacturonase activity. *Plant Pathology Journal* 9: 144-148.

Wojcik, P. (2001). "Jonagold" apple fruit quality as influenced by fall sprays with calcium chloride at high rates. *Journal of Plant Nutrition* 24: 1925-1936.

Yu J., G. Wu, W.M. Jurick II, V.L. Gaskins, Y. Yin, B. Yin, J.W. Bennett & D.R. Shelton (2016). Genome sequence of *Penicillium solitum* RS1, which causes postharvest apple decay. *Genome Announcements* 4(3):e00363-16. doi:10.1128/genomeA.00363-16.