Tomato peel (*Solanum lycopersicum* L.) colonization by the endophyte yeast *Candida guilliermondii* (Castellani) Langeron *et* Guerra

Colonización de la corteza del fruto de tomate (*Solanum lycopersicum* L.) por la levadura endófita *Candida guilliermondii* (Castellani) Langeron *et* Guerra

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ABSTRACT

During the postharvest period, fruits are lost due to attack by phytopathogens and incorrect storage and manipulation. The use of yeasts has been presented as one of the most promising strategies to resolve some of these problems. This research standardized a methodology to study, under controlled conditions, the colonization of the epidermis and subepidermal region of tomato fruit by the yeast *Candida guilliermondii*. In addition, it determined the path of yeast distribution from histological sections in tissues within 70 hours after inoculation. The results showed that *C. guilliermondii* is an endophytic yeast capable of entering through the healthy cuticle of ripe tomato fruits and colonizing apoplastic spaces without causing damage to plant tissues. The dynamics of colonization were established during the first 70 hours and the speed of yeast migration into the fruit was estimated at 0.55 μm hour^{-1}.

Key words: postharvest, histology, apoplast, biocontrol.

RESUMEN

Durante el período postcosecha de las frutas se producen pérdidas debido tanto al ataque de fitopatógenos, como al almacenamiento incorrecto y a la manipulación indebida de las mismas. El uso de levaduras se ha presentado como una de las estrategias más prometedoras para resolver algunos de estos problemas. Esta investigación estandarizó una metodología con el fin de analizar, bajo condiciones controladas, el proceso de la colonización de la epidermis y de la región subepidérmica de fruto de tomate después de ser inoculados con la levadura *Candida guilliermondii*. Adicionalmente, mediante cortes histológicos se determinó su ruta de distribución en los tejidos, durante las primeras 70 horas después de la inoculación. Los resultados mostraron que *C. guilliermondii* es una levadura endófita capaz de entrar a través de la cutícula sana del fruto de tomate maduro y colonizar espacios apoplásticos sin crear daños en los tejidos vegetales. Se estableció la dinámica de la colonización durante las primeras 70 horas, y la magnitud de la velocidad de migración de la levadura en el fruto se estimó en 0,55 μm hora^{-1}.

Palabras clave: postcosecha, histología, apoplasto, biocontrol.

Introduction

Due to the boom of the organic food industry, which is free of toxic agrochemicals, the use of yeasts has presented as one of the most promising strategies to control pathogens that produce postharvest lesions (Yu *et al*., 2007). Different authors have recommend them because they colonize the surface for long periods of time and produce extracellular polysaccharides, which favor their survival and, simutaneously, slow the growth of pathogens (Sharma *et al*., 2009). Isaeva *et al*. (2010) consider that knowledge about distribution patterns and biological properties of these yeasts will enable them to advance, from a practical point of view, in terms of solutions to problems such as fruit storage and phytopathogen control.

Nicot (2011) reviewed microorganisms used as biological controls for phytopathogens, including 14 species of *Candida* used against *Botrytis* sp. and *Monilia* sp. *C. guilliermondii* (Castellani) Langeron *et* Guerra is the anamorph (asexual form) of *Pichia guilliermondii* Wickerham; both forms have been used as biological controls of phytopathogens: *C. guilliermondii* against *Rhizopus* sp., *Botrytis cinerea* Pers.:Fr. and *Aspergillus niger* Tiegh. in the peach and grape (Zahavi *et al*., 2000; Fan *et al*., 2001); *P. guilliermondii* against *Colletotrichum capsici* (Syd.) Butler & Bisby in chilli

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fruit (Nantawanit *et al.*, 2010) and against *Botrytis cinerea* in apples (Zhang *et al*., 2011a, b).

Saligkarias *et al*. (2002a, b) found that *C. guilliermondii* secretes hydrolytic enzymes (exoglucanases and chitinases) which inhibit the development of *B. cinerea* hyphae on tomato plant stems. Zhao *et al*. (2008) detected that when applying *P. guilliermondii* and *Rhyzopus nigricans* to artificially injured tomato fruits at the same time, the former colonizes the fruit tissue, reducing the rate of infection and the size of the lesions, and stimulating responses mediated by multiple enzymes. Zhao *et al*. (2011) found that the exogenous application of *P. guilliermondii* improved the postharvest life and the quality of stored 'cherry' tomato fruit. Zhao *et al*. (2009) and Zong *et al*. (2010) determined that the use of thermal shock treatments improved the biocontrolling activity of *C. guilliermondii* against *B. cinerea* in tomato fruits.

Some of the above-mentioned papers have studied the growth of yeast on the tissues of the tomato plant and they have even determined that it is able to grow (and antagonize pathogens) in fruit tissues exposed by artificial lesions. However, they have not confirmed the possibility of colonizing tomato fruit with *Candida guilliermondii* in healthy tissues, nor have the dynamics of this process been studied.

This research sought to standardize a methodology to study the colonization of the epidermis and the subepidermal region of tomato fruit under controlled conditions, after being inoculated with the yeast *C. guilliermondii*. Consequently, it determined the yeast's path of entry and distribution in the tissues, from histological sections, within 70 h after inoculation.

Materials and methods

Plant material and disinfection protocols

Tomato fruits, without external damage and in stage six of the ripening process (Wills *et al*., 1998), were collected from tomato plants (*Solanum lycopersicum* L.) of the Chonto-Calima variety, grown under greenhouse conditions in the facilities of the "Tibaitata research center - Corpoica" (Mosquera, Colombia).

The fruits were disinfected by the following protocol: the fruits were immersed in sodium hypochlorite 0.2% for 2 min, before rinsing with sterile distilled water and immersing in 70% alcohol for 1 min. Afterwards, the fruits were rinsed with sterile, distilled water and dried with sterile absorbent paper in a laminar flow cabinet (Microzone Corporation, H6-MW-99-C30, Ottawa, Canada).

Pre-inoculation controls were carried out to verify the absence of microorganisms in the tissues. Non-inoculated fruits were cut with a sterile scalpel in a laminar flow cabinet to obtain peel samples, 3 cm^2 in area and 3 mm thick, which were placed in YGC agar (Yeast Glucose Cloramphenicol agar; Merck and Co., Franklin Lakes, NJ) Petri dishes and incubated at 25°C for 24 h.

Yeast material and inoculum preparation

The yeasts used for inoculation were isolated by the UNIDIA group (Unidad de Investigaciones Agropecuarias) from "Pontificia Universidad Javeriana" and identified as *C. guilliermondii* through biochemical methods using the API 20C AUX kit (Biomerieux Company, Hazelwood, MO). This result was confirmed using molecular techniques using PCR (polymerase chain reaction) following by RFLP (restriction fragment length polymorphism), (Orberá, 2004).

Inoculum preparation was performed by scraping colonies obtained in the YGC agar and preparing a suspension of 5 mL in saline solution 0.85% (w/v), equalized with McFarland tube 3 (Pedroza *et al*., 2006). The concentration was verified by counting with a Neubauer chamber; this suspension was added to 45 mL of YGC broth and incubated for 24 h at 27±2°C and 150 rpm, using a New Brunswick Scientific shaker gyrotory model G2 (Artisan Technology Group, Champaign, IL).

Inoculation and sampling procedures

Whole fruits were used in the first series of inoculation tests, taking one hemisphere as a control and the other one for yeast application, using three different procedures: a) by sterile swab, 200 µL of the yeast solution ($78{\cdot}10^8$ cells/mL), b) immersing the fruit hemisphere in 50 mL of the solution of the same concentration for 3 min and c) spraying with 0.3 mL of the solution. In the three cases, the inoculated fruits were stored at room temperature in sterile containers for 5 d. After that time, fruit peel samples (3 cm^2 in area and 3 mm thick) were taken of both the inoculated hemisphere and the control hemisphere. They were placed in Petri dishes with YGC agar and incubated for 3 d at a temperature of 25°C. These inoculation trials were discarded due to the frequent presence of contaminating microorganisms.

In the second series of inoculation tests, carried out in a laminar flow cabinet, samples were taken from the peel of the fruit (3 cm^2 in area and 3 mm thick) and placed individually in Petri dishes with YGC agar. Inoculation in the center of the sample was done in two different ways:

using a micropipette of 10 µL and a swab of 200 µL of yeast solution ($78{\cdot}10^8$ cells/µL). Since the use of a micropipette yielded more repeatable results, we consider this the standard procedure.

The samples inoculated with a micropipette were incubated at 25°C for time periods of 2, 5, 8, 22, 48 and 70 h (all of which had a control sample). Three replicas were made per treatment (all of which included 10 pseudoreplicas, each one a microslide with 10 serial sections; pseudoreplicas were 1,000 µm apart from each other). The controls corresponded to samples of the same fruit used for the treatments and were prepared under the same conditions, but inoculated with sterile, distilled water.

After inoculation, samples were fixed in a FAA solution (5: 90: 5 formalin, 70% ethanol and acetic acid), dehydrated for 4 h in an EtOH series (70, 90, 95% and absolute EtOH), then rinsed for 4 h in ethanol and HistoChoice Clearing Agent (Sigma-Aldrich, St. Louis, MO) mixtures at different concentrations (90:10, 70:30, 50:50, 30:70, 10:90), we ended with 100% HistoChoice®. Finally, HistoChoice® was replaced by paraffin (paraplast liquid added at 63°C, four times over the course of 12 h). Paraplast blocks, with the samples inside, were cut serially at 8 µm with a rotation microtome (820 Spencer, American Optical Company, New York, NY).

Polychromatic staining was performed on cell walls and yeast following the methodology of Kraus *et al*. (1998). However, it was modified as follows: serial cuts were spread onto microscope slides using Mayer adhesive and were dried on a hotplate with a temperature under 40°C. They were stained with 0.05% toluidine blue for 15 min, washed with distilled water and dried again at a temperature under 40°C, after which they were deparaffinized with 100% xylene, twice for 3 min and were fixed with citoresine and coverslips.

The micropreparations were photographed with a Nikon Coolpix 990 adapted to a Nikon Eclipse E200 microscope (Nikon Corporation, Tokio, Japan). Quantifications of the yeast cells were performed using quadrants of 50 μm^2, calculating the number of apoplast cells per μm^2 and discounting the intercellular spaces. All of which were executed in the following regions of interest: on and within the cuticle, in the apoplastic spaces around the epidermis and in those around the subepidermal parenchymatous cells. For quantification we used the free software ImageJ (National Institutes of Health, Bethesda, MD).

Results

The tomato fruit of the Chonto-Calima variety is characterized by an outer cuticular layer which has a thickness that varies between 6.0 and 10.9 µm. A toluidine stain gives the fruit a blue-green color and it may show anticlinal pegs which are deeply extended into the walls of the epidermal cells and have a diameter measuring between 0.8 and 1.86 µm (Fig. 1A).

The cuticle extends between the anticlinal walls of epidermal cells and, in some cases, it penetrates the first layers of subepidermal parenchyma cells, forming cuticular projections. Below are the epidermis cells with a thickness of 11.5 to 20.6 µm and walls that are stained purple. Then there are the parenchyma cells, which increase in size as they move away from the fruit surface, their walls also exhibiting purple stains (Fig. 1A).

Samples inoculated with the yeast *C. guilliermondii* look violet, oval shaped and with an average size of 3.5 x 1.2 µm (Fig. 1B). The results of the pre-inoculation controls and inoculation with sterile, distilled water indicate the absence of yeast and other endophyte organisms in the studied tissues. In addition, when evaluating the inoculated tissues, the absence of yeast cells was verified in the first 80 µm of the edges of the analyzed samples, ensuring that the yeast entered the tomato tissues through the cuticle.

According to the procedure used for staining the tissue, the yeast cells that are observed on the surface of the cuticle are those able to adhere to the tissue before being fixed with FAA. The results indicate that after 2 h, some yeasts already adhered (Fig. 2A) and some already entered the cuticle (Fig. 2B). At 8-22 h post-inoculation, an increase in the number of yeast cells adhered to the surface was observed. In addition, for storage times equal to or higher than 48 h, "clusters" of cells were formed, probably by cell division (Fig. 1D). For storage times of less than 8 h, inside the cuticle, the number of yeasts present was very low, and randomly distributed. For times higher than 48 h, the increase in the number of yeast cells was correlated with a change in distribution from random to clustered (Fig. 2B and C).

The first yeast cells were observed in apoplastic spaces around the epidermis at 8 h and around the parenchyma at 22 h. The results indicate that the yeast was distributed towards the inside of the fruit (Fig. 1C), moving between the walls of the epidermis and subsequently in the parenchyma apoplast pathway, in a random motion, forming linear

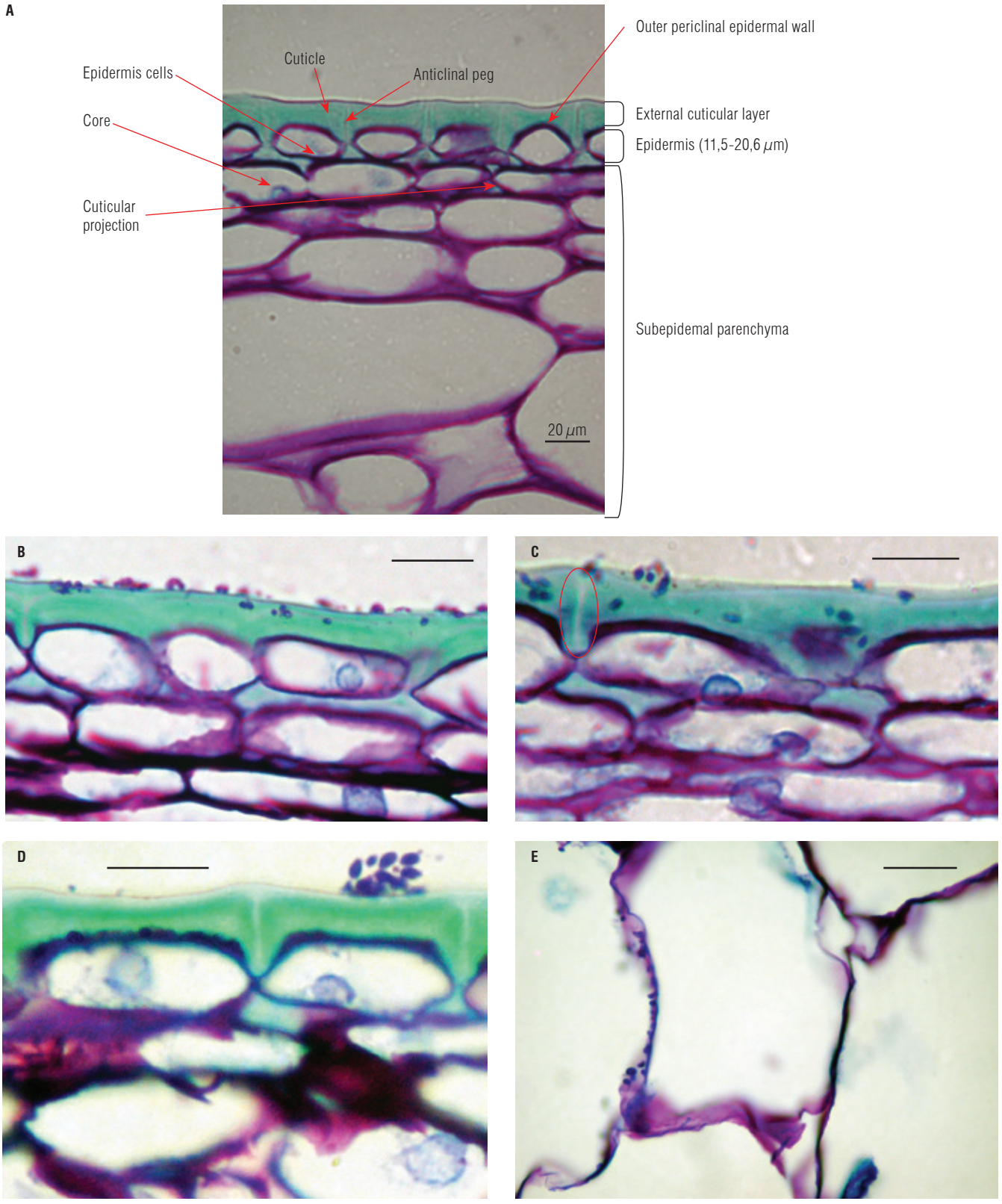

FIGURE 1. Cross-sectional images of tomato fruit epidermis stained with toluidine blue, scale bar = 20 μm. A, anatomical description of tomato fruit epidermis (control sample). Nomenclature from Matas *et al*. (2004) and Buda *et al*. (2009). B, *C. guilliermondii* yeast cells on the surface of the cuticle and inside the cuticle. Storage time: 48 h. C, *C. guilliermondii* cells on and inside the cuticle, the red area indicates an anticlinal peg and, at the top, there are yeast cells entering the fruit. Storage time: 22 h. D, clusters of the yeast on the cuticle, yeast located on epidermal cells. Storage time: 48 h. E, linear groups of yeast cells in the intercellular spaces (apoplast) of the subepidermal layer.

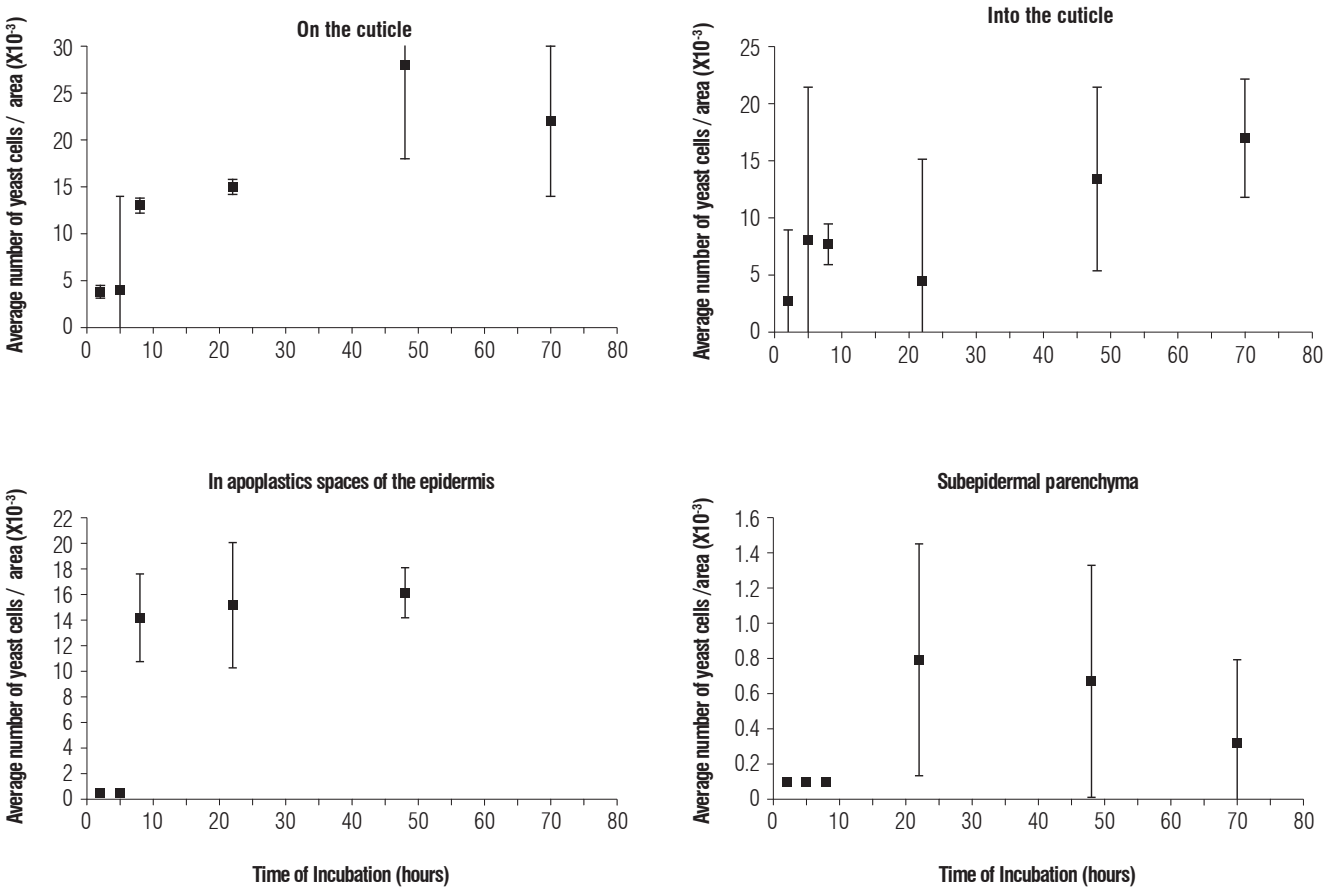

FIGURE 2. The average number of yeast cells/μm^2 in function of the time of the sample's incubation. These measures were observed in different cross-sections of the layers in the tomato epidermis and parenchyma. A, on the cuticle; B, inside the cuticle; C, in the apoplastic spaces of the epidermis; D, in the apoplastic spaces of the parenchyma. In order to count the number of yeast cells, each layer was divided into 50 μm^2 areas. In the epidermis and parenchyma, the yeast cells were only in the apoplastic areas. The bars indicate standard error.

groups (Fig. 1E) in the intercellular spaces (apoplast). Cell injury was not observed and nor was invasion into cells by the *C. guilliermondii* yeast. After crossing the barriers generated by the external cuticle, in the periclinal walls and cuticular projections, the apoplast pathway generated less resistance since the esquizogen spaces, characteristic of parenchyma, facilitated the movement of the yeast cells.

The results of Fig. 2 show a high standard error for cell densities. This is because the entry of yeast into the tomato fruit occurs randomly, so that there are many quadrants where cells are not observed; whereas once the yeast manages to cross a point, it is distributed along the less resistant routes.

Considering a straight path from the distance traveled by the yeast between the surface and its final position, we estimated an average speed of migration into ripe tomato fruit at 0.55±0.16 μm h^{-1}.

Discussion

According to our microhistological observations, together with the absence of yeast in the border of cut samples (800 μm wide), we conclude that the yeast inoculated in the ripe tomato fruits entered the fruits through the intact cuticle and distributed itself inside the fruits via the apoplastic pathway where the yeast was able to enter the intercellular spaces of the epidermis and parenchyma; conversely, other authors suggest a random entry through microdamage in tissues (Isaeva *et al*., 2010).

We found that polychromatic staining allowed for the observation of the anticlinal pegs reported by Buda *et al*. (2009), which established a diameter between 1 and 3 μm. In this research, the pegs' diameters ranged from 0.8 to 1.86 μm and yeast was observed in some of them. As a result, they are proposed as a possible explanation of how the yeast entry is facilitated. This peg structure will soon be studied by atomic force microscopy. In spite of the above,

yeast cells were also observed in the cuticle at points far from these structures (Fig. 1C).

Saligkarias *et al*. (2002b) studied the population dynamics of *C. guilliermondii* after spraying tomato stems placed in DWA agar. They found that the growth of yeast on the stems increased exponentially during the first two d, from 10^5 to 10^6 forming units of colony/portion of stem. Later, this growth slightly increased until it began to stabilize from day 13 onwards. The fact that the yeast persisted in high concentrations on the surface of the stems, between days 2 and 16, was thought by the authors to be a characteristic that makes the yeast an attractive biocontrol for *B. cinerea*. Saligkarias *et al*. (2002a, b) considered *C. guilliermondii* as an epiphytic yeast, and therefore, that measureable yeast populations are only found on the surface (not inside the tissues).

Moreover, Zhao *et al*. (2008) studied the population dynamics of *P. guilliermondii* in places of tomato fruit injury and found increases of 10^5 to 10^8 forming units of colony/injury in the first 4 d of inoculation. Nevertheless, these values are not directly comparable to Saligkarias *et al*. (2002b), because they are different forms of yeast and different organs of the plant.

In addition, it is evident that the increase in the amount of yeast is much higher in the injured tissue and this can be related to our observations concerning the ability of *C. guilliermondii* to grow inside tissues. Zhao *et al*. (2008) have pointed out that *P. guilliermondii* may be growing among the injured tissues due to the biocontrol effect it has on *R. nigricans*, and this may happen due to competition for space and nutrients. On the contrary, these authors did not seem to take into consideration the possibility that yeasts could infect intact tissues of the fruit since, in all treatments, they made lesions before implementation. Even Zhao *et al*. (2011), who applied *P. guilliermondii* to tomato fruits and determined its ability to protect them from the effect of fungi affecting postharvest, indicated that the application of antagonistic microorganisms before harvest protects injuries that occur during harvest and transport, therefore, prevents infection with pathogenic fungi, and so *P. guilliermondii* was considered an epiphytic yeast.

The strain of *C. guilliermondii* used in this research was originally isolated from internal tissues of tomato fruits and the results of this research indicate that it can colonize the internal tissues of a fruit of this species, passing through the intact epidermis. It can also grow in the apoplastic spaces, distributing itself inside them. This agrees with the definition of endophyte yeast given by Petrini (1991) and Xin *et al*. (2009). Namely, it states that endophyte yeasts are fungi formed by single cells that reproduce asexually by gemmation without a hyphal phase or a reduced hyphal phase, and can dwell in the living tissues of their host without causing apparent damage. It also complies with the condition indicated by Hallman *et al*. (1997) because this strain was isolated from vegetable material, which was superficially disinfected, but without apparent damage on the plant. Previously, only Larran *et al*. (2001) had detected endophytic yeasts in tomato leaves, specifically of the *Rhodotorula* genus.

Many studies emphasize that the interaction between endophyte microorganisms and plants is associated with the biological effects on the plants, such as biological control of soil pathogens and/or promotion of growth, as in the case of the yeast *Cyberlindnera saturnus* (Klocker) Minter (*Williopsis saturnus*), isolated from corn roots, which produces 3-indoleacetic acid and 3-indole butyric acid that promote plant growth (Nassar *et al*., 2005). In the case of *C. guilliermondii*, it is clear that it plays a role as a biological control for fruit pathogens, and its mechanisms have been studied by several authors (Saligkarias *et al*., 2002a, b; Zhao *et al*., 2011). Endophyte development capacity confers an additional advantage to *C. guilliermondii* in comparison with other microorganisms that colonize externally. Hence, not surprisingly, it has been selected as one of the top two biocontrols for *Botrytis*, *Aspergillus* and *Rhizopus* in grapes, from 129 different strains of diverse microorganisms all considered epiphytic by Zahavi *et al*. (2000).

In order to research the nutritional changes in fruits inoculated with the yeast *C. guilliermondii*, new studies are necessary. Moreover, it is vital to employ other techniques to study possible changes in the fruit and fruit surface before yeast is inoculated. This could contribute to the knowledge of the interaction between fruit and yeast.

Conclusions

The methodology in this research shows a new way of studying the dynamics of colonization of the yeast *C. guilliermondii* in plant tissues. The results demonstrate that *C. guilliermondii* is an endophytic yeast, capable of entering through the healthy cuticle of the ripe tomato fruit and colonizing the apoplastic spaces without causing damage to plant tissue cells. This makes an important contribution to the knowledge that we need to use this yeast for bio-control purposes; and improving and increasing the storage time of tomato fruits. Finally, we also calculated

the speed magnitude of yeast migration during the first 70 h at 0.55±0.16 μm h^{-1}.

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