Antifungal activity of vanilla juice and vanillin against *Alternaria alternata*

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**ABSTRACT**

Vanilla juice has been shown empirically to have antifungal activity against some fungal strains; however, there are no activity reported against *Alternaria* genre. In this work, the chemical profile of vanilla juice was obtained and its antifungal activity against fungal strains from the family Pleosporaceae, isolated from sorghum- and barley-diseased plants, was tested. The strains were identified as *Alternaria alternata* by their molecular and morphological characteristics. The vanilla juice characterization from *Vanilla planifolia* pods showed the presence of vanillin, vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, guaiacol, glucovanillin, vanillyl alcohol, and furfural. Vanilla juice showed a fungistatic effect against all *A. alternata* strains tested in this study and increased the lag time from 50 to 112 h, and no conidia were produced. This result indicates the possible application of vanilla juice as an alternative to control agricultural crops such as barley and sorghum in Mexico.

1. Introduction

Vanilla extraction is an artisanal process, consisting of four stages: killing, sweating, drying, and conditioning the vanilla pods (Dignum, Kerler, & Verpoorte, 2001a). Vanilla juice is obtained during the sweating process and it is composed mainly of four aromatic compounds: vanillin, vanillic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid (Havkin-Frenkel, French, Graf, Pak, & Frenkel, 2004; Rao & Ravishankar, 2000). Each sweating cycle lasts between 36 to 48 h (De la Cruz et al., 2009) and from 4 to 13 cycles can be undertaken, depending on the vanilla pods maturity (Havkin-Frenkel et al., 2004; Purseglove, Brown, Green, & Robins, 1981; Rosado, 2006). After sweating, vanilla pods are exposed to the sun or are placed on wooden pallets in the conditioning area, depending on weather conditions (Rosado, 2006). During water removal, the pressure exerted by the vanilla pods weight facilitates juice collecting (Rosado, 2006). The vanillin molecule is recognized as safe and is used as an antimicrobial (Cerruti & Alzamora, 1996). Vanillin inhibits bacteria (Cava, Taboada, Valverde, & Marín, 2012; Delaquís, Stanich, & Toivonen, 2005; Fitzgerald et al., 2004) and fungi growth (Lopez-Malo Cerruti & Alzamora, 1996; Kim et al., 2014; Rivera-Carriles, Argaiz, Palou, & López-Malo, 2004). Vanillin changes community structures of *Fusarium* and decreases the number of bands as confirmed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis, and quantitative PCR (Zhou, Jia, Ge, & Wu, 2018).

Therefore, vanilla juice and vanillin can be considered agrochemicals for the control of pathogens resistant to different synthetic fungicides (Diaz et al., 2011). *Alternaria alternata* is the causal agent of diseases, like barley black point (Zare, 2013) and gray leaf spot in sorghum (Thomas, 1991). These diseases reduce the commercial value of cereals, causing farmers to lose between 15 and 90% of untreated field-grown seeds (Lipps, 1998; Mathre, 1997; Zare, 2013). In this study, we characterized the vanilla juice from *Vanilla*...
planifolia pods and studied its antifungal effect against *A. alternata* strains isolated from sorghum and barley plants.

2. Materials and methods

2.1. Fungal cultures

*A. alternata* fungal strains used in this study were isolated from diseased plants of sorghum (*Sorghum* sp.) and barley (*Hordeum vulgare*), collected at different times (Table 1). To isolate fungi, some infected sorghum fragments and barley plants were placed in a moist chamber and incubated in darkness at 25°C for 3 days. After that, each fungus was isolated and subcultured on potato dextrose agar, PDA (Sigma, St. Louis, MO), at room temperature for 7 days. Fungal strains were maintained in PDA medium at 4°C and the spore suspension at −8°C.

2.2. Molecular and morphological characterization

Strains isolated (JCP13, JCP25, JCP31, JCP32, JCP49, JCP56) were characterized molecularly and the sequences were deposited in the GenBank. Strain ITV3 was donated and characterized in the Instituto Tecnológico de Veracruz (ITVer). Morphological descriptions are based on comparisons of *A. alternata* (Ariyawansa et al., 2015; Lawrence, Rodonto, & Gannibal, 2016) augmented by new observations, as noted. The standard medium used to assess morphology and growth rate was PDA. The morphology, colony color, and diffusing pigment in the agar were recorded. Twenty-five measurements of conidia and conidiophores were made. Measurements of the characters were taken from images using the software IOS 7, 8-megapixel iSight camera, iPhone 5S version Microsystems (Hollyhill, Cork, Republic of Ireland). Scanning electron micrographs were taken with the Cryo Transfer System (Jeol, model IT300) at the Escuela Superior de Aparc (ESAP-UAEH).

2.3. Vanilla juice extraction

Vanilla juice was obtained from the sweating stage during the curing process of vanilla in Gutierrez Zamora, stated of Veracruz, Mexico. Vanilla juice was collected in sterile bottles 2 days after initiating the sweating cycles from a batch containing 1000 kg of vanilla pods. Samples were kept on ice during their transport to the laboratory.

2.4. pH measurements

The pH measurements were performed in the vanilla juice using a potentiometer (pH 510 Series Benchtop Meter Oakton model 00702–93) with a magnetic agitator. The measurements were performed in triplicate.

2.5. Chromatographic profile

The chromatographic profile was performed according to the technique proposed by Pérez-Silva et al. (2006), using an HPLC (Varian ProStar model 240) equipped with a UV detector (Waters model 2487), 230 nm wavelength, and a C18 column (Microsorb TM-MV). The mobile phase was a mixture of methanol-acidified water that was prepared as follows: the HPLC grade water (J.T. Baker) was acidified with 0.1 mol L\(^{-1}\) phosphoric acid (HYCEL), filtered through a 0.45-μm membrane, stirred for 5 min, and then sonicated (West prime Systems) during 30 min. HPLC grade methanol (J.T. Baker) was subjected to the same treatment. For the whole test, a flow of 0.7 mL min\(^{-1}\) was used. Vanilla juice samples were diluted with the mobile phase and filtered through a membrane (0.22-μm) before being injected into the HPLC. To determine glucovanillin, vanillyl alcohol, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and vanillin acid a column was used, with temperature at 35°C, a ratio phase (methanol-acidified water, 78:22, v/v), and a pressure of 5.269 × 10\(^6\) Pa. Vanillin and guaiacol were identified using a column temperature at 30°C. A ramp to the mobile phase was applied also, starting with a proportion of 78:22 decreasing 3 mL every 2 min until reaching a proportion of 65:35. The pressure ranged from 7.295 × 10\(^6\) Pa to 9.727 × 10\(^6\) Pa. Furfural was determined using a column temperature at 30°C, an initial phase with a ratio of 90:10, and a decreasing ramp of 2 mL every 3 min to reach a ratio 78:22. Pressure ranged from 5.269 × 10\(^6\) to 5.472 × 10\(^6\) Pa. Calibration curves were performed with an external control using: vanillic acid (VA; Fluka), vanillyl alcohol (VAL; Fluka), guaiacol (GYL; Fluka), vanillin (V; Sigma), *p*-hydroxybenzoic acid (BHA; Fluka), *p*-hydroxybenzaldehyde (PHB; Fluka), and furfural (FUR; Sigma-Aldrich). Glucovanillin (GLU) was extracted with the method reported by Odoux (2000) and was donated by the CIRAD Institute (France).

2.6. In vitro antifungal assays

The antifungal effect of vanilla juice and commercial-grade vanillin was evaluated in *vitro* on PDA medium. A volume of 2 μL with 2.0 × 10\(^6\) spores was deposited in the center of each Petri dish, containing PDA medium as a control, PDA with vanilla juice (adjusted to 250 mg L\(^{-1}\) of vanillin), and PDA with commercial-grade vanillin. Experiments were performed at a temperature of 25°C during 7 days. The pure vanillin (Sigma) was used at different concentrations (250, 500, 750, 1000, 1250, and 1500 mg/L). Radial growth (mm) was measured every 24 h during 8 days. The tests were performed in triplicate and the results were analyzed using the STATISTICA software (Hill & Lewicki, 2007).

3. Results and discussion

3.1. Isolation and morphological identification

The pleomorphic genus *Alternaria* (Lawrence, Gannibal, Peever, & Pryor, 2013) was named by Lawrence et al. (2013), it comprises around 60 species that have small spores and include *A. alternata*, *A. arborescens*, *A. gaisen*, and *A. tenuissima* (Lawrence et al., 2016).
Alternaria spp. are well known to produce many secondary metabolites-related toxins (Christensen et al., 2005; Frisvad, Andersen, & Thrane, 2008). These metabolites are responsible for many plant pathogens with or without being host specific (Markham & Hille, 2001; Wolpert, Dunkle, & Ciuffetti, 2002) and with mycotoxins implicated in food contamination (Fernández-Cruz, Mansilla, & Tadeo, 2010; Ostrov, 2008). In this sense, small-spored organisms, such as A. alternata, also play an important role in inducing and causing some pathologies in humans and plants (Salo et al., 2006; Singh, Gupta, & Sharma, 2014).

In this study, we isolated seven fungal strains from diseased plants; six strains were isolated from barley (JCP13, JCP25, JCP31, JCP32, JCP49, and JCP56) and one strain from sorghum plants (ITV3). Colonies in PDA medium showed rapid growth, the fungi reached a diameter of 37–40 mm in 7 days, at 25°C (Figure 1(a)). The initial growth was hairy, with a gray color that later changed in the center of the colony acquiring a darker tone, with more or less intense tones but still with gray borders. The reverse of the colonies showed a black color (Figure 1(b)). Hyphae had a filamentous shape with simple conidiophores, septa, from their end muriform conidia were formed, of a grayish brown color, and transverse and vertical multi-septa in an irregular arrangement (Figure 1(c)). The new conidia are formed by gemmation from the apical cell, giving rise to a long chain of more than 10 conidia (Figure 1(d,e)). The primary conidiophores can be curved or straight, from short to very large in size, simple or branched with one or more terminal conidiogenous loci.

Asexual morphological characteristics on PDA medium showed abundant conidia that were small to moderate in size, (20-)20.68–29.42(−38) × (8-)18.18–9.76(−11) mm (av. = 25.04, SD = 4.37, n = 25; av. = 8.97, SD = 0.79, n = 25), obclavate (Figure 2(a)), long ellipsoid or ellipsoid (Figure 2(b,c)), from 3 to 7 transversal septa (Figure 2(d)). A slight constriction was observed also in some septa, with 1 or 2 longitudinal septa in one or a few transversal divisions (Figure 2(e)). Based on molecular techniques, the phenotype descriptions and the measurements described, the strains were identified as Alternaria alternata.

The results observed in this study are within the range of observed values for other A. alternata strains reported by other authors (Ariyawansa et al., 2015; LawRENCE et al., 2016) and lower than those reported by (Nagrale, Gaikwad, & Sharma, 2013; Ramjugathesh & Ebenezar, 2012) for the maximum length of conidia.

3.2. Vanilla juice characterization

3.2.1. pH measurement

Vanilla juice had similar organoleptic characteristics to the vanilla extract, with a predominantly acidic pH, in a range between 4.4 and 5.6. The low pH was associated with the organic acids present, such as acetic, propionic, butyric and isobutyric, propanoic acids, and others (Pérez-Silva et al., 2006), the maturity degree of vanilla beans, the sweating cycles (8 to 13), and the harvest time of the juice (Rosado, 2006).

This pH result corresponds to the pH reported by Pacheco (2009) for green vanilla extract and vanilla cured extract,
5.12 ± 0.19 and 5.08 ± 0.24, respectively. Vanilla juice is a byproduct obtained from approximately a ton of vanilla beans; therefore, the juice is a concentrated product containing large amounts of the chemical compounds present in vanilla beans. The vanilla juice had an oily or viscous consistency and a brown color. The juice was collected during the first cycles of sweating (18–20 h) as inferred by the amount produced (Tapia et al., 2011). At this stage, the vanilla bean cells destruction is accelerated, which leads to the formation of vanillin and the generation of large amounts of chemical compounds (Purseglova et al., 1981; Romero, 2003; Walton, Mayer, & Narbad, 2003). During the sweating phase, temperatures reached up to 65°C, which may facilitate the release of simple aromatic compounds and other polymers such as waxes, resins, gums, and essential oils (Ranadive, 1994; Rosado, 2006).

3.2.2. Chemical compounds in vanilla juice

Chromatographic characterization showed the same profile as the cured vanilla bean extract obtained from a traditional curing process (Figure 3(a)). The chemical concentration of the compounds in vanilla juice was evident in the chromatograms and was higher than those reported in the cured vanilla (Figure 3(b)). Furfural, guaiacol, and vanillyl alcohol compounds have been reported as traces in the cured vanilla extract (Pérez-Silva et al., 2006), and their presence in the vanilla juice was evident in the chromatograms and was higher than those reported in the cured vanilla (Figure 3(b)). Furfural, guaiacol, and vanillyl alcohol compounds have been reported as traces in the cured vanilla extract (Pérez-Silva et al., 2006), and their presence in the vanilla juice was evident in the chromatograms and was higher than those reported in the cured vanilla (Figure 3(b)).

Chemical compounds identified in the juice are summarized in Table 2.

The presence of glucovanillin and vanillyl alcohol in the juice was very important as vanillin precursors. The results obtained show that conversion of vanillin does not reach 100% in cured vanilla beans.

The four main aromatic compounds in the juice are due to their formation during the first 24 h after the curing process had started (Havkin-Frenkel et al., 2004). Dignum, Kerler, and Verpoorte (2001b) confirmed the presence of vanillin and vanillic acid in the first sweating cycles, but the glucovanillin presence has been reported in the ninth cycle of sweating at concentrations of up to 0.08 g 100 mL−1. Other authors reported concentrations of p-hydroxybenzoic acid between 0.019 g 100 mL−1 and 0.034 g 100 mL−1 (Gassenmeier, Riesen, & Magyar, 2008). Vanillin concentrations ranged from 0.10 g 100 mL−1 to 0.61 g 100 mL−1. Odoux (2000) reported that vanillin concentration increased with each sweating cycle at around 0.6 g kg−1 in dry matter (DM), in the green pod, and at a concentration of 0.2 g kg−1 DM in cured pods. Dignum et al. (2001b) reported the presence of guaiacol in the cured vanilla extract obtained during the killing stage under controlled conditions. Guaiacol showed higher concentration on the first day of the killing stage until reaching a maximum conversion on the fourth day, which explains the changes in concentrations found in this research. Farmers collected the juice during the first sweating cycles because in this step the vanilla beans lose up to 40% water (Havkin-Frenkel et al., 2004; Purseglova et al., 1981). Unlike the later sweating cycles, where there is less dehydration and the amount of juice produced is minimal (Rosado, 2006).

During the sweating stage, the temperature may reach 65°C or higher (Rosado, 2006) and can form degraded
products such as furfural (Rao & Ravishankar, 2000). The vanilla juice showed different concentrations in the five batches studied. In general, the chemical compounds identified in the juice had a higher concentration (except vanillin) than in the cured vanilla beans (Pérez-Silva et al., 2006; Rosado, 2006). During the sweating and drying stages, the development of aromatic chemical compounds increases and can be dragged with water (Dignum et al., 2001a; Rosado, 2006).

### 3.2.3. Antifungal action

The vanilla juice was adjusted to 250 mg/L of vanillin concentration and was compared to commercial-grade vanillin, results did not show antifungal effect at the concentration tested. This can be attributed to the vanillin concentration tested and the difference in the pH value at which the tests were conducted, since the pH of the juice was 4.4; whereas the PDA medium in which vanillin is contained has a pH of 5.0 (Delaquis et al., 2005). The decrease in radial growth suggests that the fungus was unable to maintain a positive tannin pressure in the mycelium to counteract the effect of the chemical compounds in the growth medium (Tijerina-Ramírez, Lira-Méndez, Moreno-Medina, González-Prieto, & Mayek-Pérez, 2014) by limiting the availability of free water for growth (Harris, 1981). *A. alternata* is a fast-growing fungus of wet environments (Pontón, Moragues, Gené, Guarro,
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A. alternata (46.9%),
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, and
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Calotropis gigantean
A. alternata (Vargas, Gamboa, Medina, & Pérez,
(2014)). Other authors (Tapwal, Nisha Garg, Gautam, & Kumar, (2011)) reported a 50% inhibition of growth by an aqueous leaf extract of Parthenium hysterophorus against Alternaria solani. This effect was explained by the release of some phytotoxic substances such as feluic acid, caffeic acid, vanillic acid, anisic acid, chlorogenic acid, p-coumaric acid, p-hydroxybenzoic acid, parthenin, ambrosin, and coronopin (Prusti, Mishra, Sahoo, & Mishra, (2008)). Fitzgerald, Stratford, Gasson, and Narbad (2005) analyzed the structure-function of the vanillin molecule and its antifungal properties and of six analogous chemical com-
Figure 4. Commercial-grade vanillin concentration effect on radial growth rate on A. alternata strains at three different times.
Figura 4. Efecto de la concentración de vainillina de grado comercial sobre la tasa de crecimiento radial de la cepas de A. alternata en tres tiempos diferentes.

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against *A. ochraceus* strain, this effect was achieved at pH 3 and ≤25°C or pH 4 and ≤15°C.

Lopez-Malo, Alzamora, and Argaiz (1998) reported that the germination time and radial growth rate of *Aspergillus flavus*, *A. niger*, *A. ochraceus*, and *A. parasiticus* was affected significantly when vanillin concentrations of 500, 750, and 1000 mg/L were used at pH 3.0 and 4.0 with aw 0.98. In addition, Delaquis et al. (2005) reported that with the increase of pH, the vanillin effect goes from bacteriostatic to bactericidal. This is because the organic antimicrobial agents, including phenolic acids, are more active in a dissociated state, because the pH is close to the pKa, explaining the bactericidal effect of vanillin (pKa 7.4) against some species of *Listeria* at pH 7.0.

Vanillin concentrations used in this research were based on the research reported by Lopez-Malo et al. (1997, 1998). However, the results were not consistent with expectations. These results may be due to the fact that the vanillin
4. Conclusion

Vanilla juice shows a fungicidal effect against all A. alternata strains studied and increased the pathogen’s lag time from 50 to 112 h. A synergistic, additive, or antagonistic effect by the other chemical compounds present in vanilla juice was observed as compared to commercial vanilla. This vanilla juice can be concentrated and could be used in programs to evaluate a large number of extracts as natural antifungal products. The information obtained in this research would be useful to obtain the four aromatic compounds reported in the cured vanilla bean (vanillin, vanillic acid, p-hydroxybenzoic acid, and p-hydroxybenzoic acid) and precursors of vanillin (glucovanillin and vanillic acid). The presence of these compounds in the vanilla juice decreases its yield concentration in the cured vanilla beans at the end of the processing. Vanilla juice could be used as an alternative option for the control of fungi responsible for diseases in plants of commercial interest in Mexico.

Disclosure statement

No potential conflict of interest was reported by the authors.

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