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Effects of boiling and oil or vinegar on pickled jurubeba (Solanum paniculatum L.) fruit

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Jurubeba fruit, raw and thermally processed for different periods of time, were preserved in soybean oil or vinegar and evaluated for physical characteristics, phytochemicals, antioxidant capacity and polyamines. The loss of green color in many vegetables after cooking is a frequent problem that affects the quality of pickled foods, and chlorophyll content is a relevant parameter to assess the quality. Data showed that a 20 min cooking treatment maintained the best fruit quality and no change in the chlorophyll content occurred. The thermal processing caused no increase in the carotenoid and flavonoid content as compared to the raw fruits, but caused an increase in the phenol content. At a cooking time of about 10 min, the antioxidant capacity increased. Cooking time did not cause significant differences in the content of isoorientin, rutin and caffeic acid. Spermine and spermidine contents were lower after 20 min of boiling. Jurubeba that was preserved in vinegar showed a lower pH and putrescine level, regardless of the cooking time used, whereas the use of oil caused an increase in carotenoids and antioxidant capacity.

Key words: Thermal processing, antioxidants, polyamines, phytochemicals, Solanaceae.

INTRODUCTION

Globally, significant improvements have been made through studies of regional dietary habits and considerable inter- and intra-country variability (Kearney, 2010). Much important information has been provided by research that has focused on non-conventional foods that are consumed together with staple foods to improve taste and nutritional quality. These non-conventional foods include Solanum paniculatum L. (Solanaceae), popularly known as “Jurubeba”, which is widely used in folk medicine as a tonic and antipyretic agent (Santos et al., 1988). This plant is native to northern and northeastern regions of Brazil and produces a dark-green fruit that is used for culinary purposes. S. paniculatum fruits are mainly consumed after cooking, with rice or as pickles prepared either in oil or vinegar. Heat treatment is the main cause of change in...
the content of natural antioxidants in food (Kaur and Kapoor, 2008). However, the cooking process can contribute to the formation of new compounds, or promote the easier extraction of molecules from the cell matrix. In fruits and vegetables, many bioactive compounds, such as polyphenols, carotenoids and polyamines, are present in variable concentrations. The plants of the genus *Solanum* contain steroidal saponins, glycoalkaloids and flavonoids—secondary metabolites that are important for the natural defenses of plants (Ramos et al., 2012). An evaluation of some plants in relation to their antioxidant potential (the chemical constituents that assist in free-radical scavenging), such as polyphenols, vitamins, alkaloids, triterpenes, sesquiterpenes and other molecules has been the object of several studies (Ramos et al., 2012).

The most common polyamines (PAs) in fruits and vegetables are putrescine, spermidine and spermine—compounds that are frequently affected by the cooking process and heat treatments (Rossetto et al., 2015). Some fruits are rich in putrescine (Lima et al., 2008), whereas green vegetables are richer in spermidine (Valero et al., 2002). The occurrence of polyamines in jurubeba has not yet been described and their quantification is necessary.

Information regarding the nutritional composition of *S. paniculatum* fruits, in natura or after cooking, is scarce or absent. Thus, the aim of this research was to evaluate the effects of thermal processing on the quality of *S. paniculatum* fruits by assessing the content of vitamin C, pigments, total phenols, total flavonoids and characterizing some polyphenols by HPLC, and analyzing total antioxidant capacity in raw and heat-treated samples. In addition, the effect of preservatives such as oil or vinegar on pickled fruits was established.

**MATERIALS AND METHODS**

**Reagents and samples**

All chemicals and flavonoids standards (isoorientin, rutin or caffeic acid) were purchased from Sigma Co. (St Louis, MO, USA). *S. paniculatum* fruits were harvested in February 2014, from a farm located in Caceres, Mato Grosso State, Brazil, (16°04'14''S latitude, 57°40'44''W longitude and 118 m altitude), and were transported to the laboratory. This species was identified by the botanical characteristics and a voucher (BOTU 027535, BOTU 027536 and BOTU 027537) was previously deposited at the UNESP Herbarium, in Botucatu, São Paulo State, Brazil (Vieira Junior et al., 2015). A homogeneous and representative sample, whole fruits with intact peel and in the same maturity stage, was selected and sanitized in a commercial chlorine bleach solution.

**Cooking process and the preparation of pickles**

To obtain cooked samples, 150 g fruit was placed in stainless steel pans with 1 L boiling distilled water and was cooked for 10, 20, 30 or 40 min at atmospheric pressure. After this procedure, the remaining water was drained and the fruits were cooled at room temperature.

Raw and cooked fruits were pickled in sterile flasks filled with 2.5% NaCl in two different preservatives: soybean oil or alcohol vinegar. After preparation, the pickle bottles were sealed and stored at room temperature (23 ± 2°C) for 20 days, as shown in Figure 1.
Soluble solids, pH and titratable acidity

The soluble solids were analyzed using a digital refractometer (Atago, PAL-1 model). The pH was determined by a potentiometer (model Q Quimis, - 400A). Titratable acidity was determined in gram citric acid 100 g⁻¹, by titration, using 2 g of ground product and 20 mL of distilled water, as previously reported (Bett-Garber et al., 2015).

Vitamin C

The amount of vitamin C was determined using 2 g fruit ground in liquid nitrogen and diluted in 10 mL oxalic acid. This method is based on the reduction of 2,6-dichlorophenolindophenol dye by ascorbic acid (Zenebon et al., 2008).

Carotenoids, chlorophyll, total phenols and flavonoids

Raw, cooked and pickled samples were ground with liquid nitrogen and stored at -80°C, until analysis. The determination of carotenoids and chlorophyll (a and b) was carried out using the method of Sims and Gamon (2002). From each sample, 100 mg was homogenized in a mini-turrax (Marconi, Brazil) with 3 mL cold acetone/Tris-HCl (0.2M, pH 7.8, 80:20, v/v) solution for 1 min. All procedures were conducted on ice and were protected from light. The samples were then centrifuged at 2,000 g for 5 min and the supernatant was immediately used for the determination of pigments, using a UV/VIS spectrophotometer (Amersham Pharmacia Biotech). Total chlorophyll was obtained by the sum of chlorophyll a and b. The absorbance values were converted into µg total carotenoids g⁻¹ based on the formula:

\[ \text{Carotenoids} = \{A_{670}\times17.1\times(\text{Claro+Clb})-9,479\times\text{anthocyanin}\}/119.26^* \]
\[ \text{Chlorophyll } a = 0.01373^* (A_{663})-0.000897 (A_{537})-0.003046 (A_{647}) \]
\[ \text{Chlorophyll } b = 0.02405^* (A_{475})-0.004305 (A_{537})-0.005507 (A_{647}) \]

Total phenols were determined based on the method of Singleton and Rossi (1965) using the Folin-Ciocallet reagent. Fresh powdered samples (100 mg) were homogenized in a mini-turrax (Marconi, Brazil) with 5 mL acetone:water (50:50 v/v). After 20 min in an ultrasonic bath (Eco-sonics, Ultronique), samples were centrifuged for 10 min at 6,000 g at 5°C. The supernatant was removed and retained. The extraction process was performed once and the supernatants were combined. Absorbance was measured at 725 nm and the results were expressed in mg gallic acid equivalents g⁻¹ fresh weight.

For flavonoid analysis, the samples were extracted in methanol. After 60 min in an ultrasonic bath, the samples were centrifuged at 6000 x g (HeitichZentrifugen, MikR0 220R) for 10 min and the supernatant was collected. Extraction was conducted in the pellet twice and supernatants were combined and analyzed for the total flavonoid (Popova et al., 2004). The complete process was carried out in the absence of light. The results were expressed in mg quercetin g⁻¹ fresh weight.

Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant capacity was determined using the methodology proposed by Brand-Williams et al. (1995), adapted by Rossetto et al. (2009). The results were expressed in uM of Trolox equivalents µg g⁻¹ sample (TEAC). The extract was obtained from 100 mg pulverized fresh samples in liquid nitrogen, homogenized in a mini-turrax (Marconi, Brazil) and kept for 15 min in an ultrasonic bath (Eco-sonics, Ultronique) with 3 mL ethanol. Subsequently, the samples were centrifuged for 10 min at 6,000 g at 5°C and after 30 min, the absorbance was measured at 517 nm, in a UV/VIS spectrophotometer (Amersham-Pharmacia-Biotech).

Thin layer chromatography of polyamines (PAs)

Polymamines were analyzed as described by Flores and Galston (1982), with modifications by Lima et al. (2008). Solanum panicatum fruits were homogenized in cold perchloric acid (5% v/v) for 1 h and were centrifuged (10000 x g, HeitichZentrifugen, MikR0 220R) for 30 min at 4°C. Then, 4.5 mol L⁻¹ Na₂CO₃, containing 18.5 mmol L⁻¹ dansyl chloride in acetone (Sigma, 95%) was added to the supernatant. The reaction was carried out at room temperature and was protected from light for 16 h. Then, 0.87 mol L⁻¹ proline (Sigma, 95%) was added, and the samples were maintained at room temperature for 30 min. Toluene (Sigma) was used to extract the dansylated PAs. Aliquots (20 µL) were applied manually with a Hamilton syringe (50 µL) onto activated (1 h at 110°C before use) glass plates (Adamant® Silica gel 60G, 0.25 mm (20 × 20cm). Machery-Nagel) and were separated in a thin layer chromatography (TLC) developing tank, using chloroform : triethylamine (7.5:1) as a mobile phase. The plate was allowed to dry at room temperature (22 ± 2°C), and was then dried with a hair dryer to remove excess solvent. Putrescine (Put) (Sigma, 98%), spermidine (Spd) (Sigma, 99%) and spermine (Spm) (Sigma, 99%) were used as standards. The entire procedure was monitored under UV light at 254nm. Free PAs were quantified by comparison against standards by fluorescence emission spectroscopy (excitation at 350 nm and emission at 495 nm), in a Video Documentation System, using the Image Master 2.0 Software (Amersham Pharmacia Biotech 1996). The calculation of the quantitative analysis was performed based on the area obtained from the standards and the samples. The free PAs content was expressed as nmol g⁻¹ fresh weight (FW).

High-performance liquid chromatography (HPLC) analysis of flavonoids

Samples were extracted, filtered (Millipore 0.22 µm filter) and used for flavonoid analyses according to a previous report method (Escarpa and González, 1996). Briefly, 20 µL sample was injected into a Thermo Scientific Dionex UltiMate 3000 system (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary pump, an Ultimate 3000RS auto sampler and a diode array detector (DAD-3000RS). Flavonoids were separated on an Ace C18 (4.6 × 250 mm; 5µm) column at 25°C. Analysis was monitored at 280 nm and peak integration and calibrations were performed between 210 and 350 nm using Dionex Chromelone software. The flow rate was 1.0 mL min⁻¹ and the mobile phase consisted of methanol (solvent A) and 0.01M phosphoric acid. The system was run with the following gradient elution program: 0-5 min, 0.5% A, 5-10 min, 50% A, 10-15 min, 70% A, 15-20 min, 80% A, 25-30 min 100% A and to 30-35 min 5%. Flavonoids were quantified by determining peak areas under the curve in the HPLC calibrated against known amounts of standards. Identification of each peak in unknown peak was confirmed by the retention time and characteristic spectra of the standards. The interassay coefficient of variation (CV) was 3.7% (n = 15) and the intra-assay CV was 4% (n = 8).

Statistical analysis

All the results are given as means ± standard deviation. Differences between variables were tested for significance using a one-way ANOVA procedure, followed by Tukey’s test, at a significance level of p<0.05.
Table 1. pH, soluble solids (“Brix”), titratable acidity (g citric acid 100 g⁻¹) and ratio (SS/TA) of jurubeba raw and subjected to different boiling times (10, 20, 30 and 40 min) and types of preservatives (oil and vinegar).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Oil</th>
<th>Vinegar</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>5.58 ± 0.07abA</td>
<td>5.58 ± 0.07aA</td>
</tr>
<tr>
<td>10</td>
<td>5.45 ± 0.03abA</td>
<td>3.94 ± 0.02bB</td>
</tr>
<tr>
<td>20</td>
<td>5.63 ± 0.06bA</td>
<td>3.90 ± 0.00bB</td>
</tr>
<tr>
<td>30</td>
<td>5.58 ± 0.10abA</td>
<td>3.92 ± 0.02bB</td>
</tr>
<tr>
<td>40</td>
<td>5.52 ± 0.10abA</td>
<td>3.90 ± 0.01bB</td>
</tr>
<tr>
<td>TA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>0.14± 0.01abA</td>
<td>0.14± 0.01abA</td>
</tr>
<tr>
<td>10</td>
<td>0.11± 0.01aB</td>
<td>0.33± 0.07aA</td>
</tr>
<tr>
<td>20</td>
<td>0.07 ± 0.01bB</td>
<td>0.28± 0.00aA</td>
</tr>
<tr>
<td>30</td>
<td>0.06± 0.00bB</td>
<td>0.32± 0.01aA</td>
</tr>
<tr>
<td>40</td>
<td>0.10± 0.00bB</td>
<td>0.32± 0.00aA</td>
</tr>
<tr>
<td>SS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>15.33 ± 0.7abA</td>
<td>15.33 ± 0.7abA</td>
</tr>
<tr>
<td>10</td>
<td>18.7 ± 2.7abA</td>
<td>9.9 ± 0.6abB</td>
</tr>
<tr>
<td>20</td>
<td>21.3 ± 5.1abA</td>
<td>9.87 ± 0.8abB</td>
</tr>
<tr>
<td>30</td>
<td>19.63 ± 1.7abA</td>
<td>9.03 ± 0.3abB</td>
</tr>
<tr>
<td>40</td>
<td>18.73 ± 3.7abA</td>
<td>9.27 ± 1.1abB</td>
</tr>
<tr>
<td>SS/TA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>107.95 ± 11.59cA</td>
<td>107.95 ± 11.59acA</td>
</tr>
<tr>
<td>10</td>
<td>174.00 ± 31.43cA</td>
<td>31.03 ± 8.12bB</td>
</tr>
<tr>
<td>20</td>
<td>283.61 ± 31.04cA</td>
<td>35.05 ± 2.33bB</td>
</tr>
<tr>
<td>30</td>
<td>295.08 ± 6.07cA</td>
<td>27.47 ± 2.01bB</td>
</tr>
<tr>
<td>40</td>
<td>176.59 ± 36.29cA</td>
<td>28.74 ± 0.99bB</td>
</tr>
</tbody>
</table>

*Within columns means followed by different lowercase letters and upper case on lines are significantly different based on Tukey’s ANOVA test (P < 0.05).

RESULTS AND DISCUSSION

Cooking time influenced the quality parameters (pH, soluble solids and soluble solids/titratable acidity) in S. paniculatum fruit that were conserved in oil (Table 1). The results showed a slight variation in quality parameters for fruits that were pickled in vegetable oil and cooked for different lengths of time. The highest values were observed for fruits boiled for 20 min. Jurubeba fruit that were pickled in vinegar, which has a high acidity, exhibited a lower pH than those preserved in oil. Besides consumer acceptance, the pH affects many chemical processes, such as protein properties (denaturation), enzymatic activity and also the growth of microorganisms (Stippl et al., 2004).

This effect on pH was reflected in the titratable acidity of samples preserved in vinegar, as well as in oil. Fruit that were boiled for 20 min and preserved in oil showed higher levels of SS, although no difference was observed at other cooking times. When pickled in vinegar, the values for SS after 20 min were similar to those at 10 min. The ratio of SS/TA increased with the use of oil as a preservative and with the cooking time, as compared to that of raw fruits. However, this ratio decreased proportionally with an increase in cooking time in fruits preserved in vinegar.

In the present study, chlorophyll and carotenoid levels decreased after cooking as compared to raw fruits (Figure 2A and B). The loss of green color in many vegetables after cooking is a frequent problem that affects the quality of many canned fruits and vegetables. At room temperature, chlorophylls (a and b) are stable, but when temperatures above 50°C are used, chlorophyll levels can be affected (Andrés-Bello et al., 2013). The decreased levels of chlorophyll following heat treatment is due to the conversion of chlorophyll to pheophytin, which is attributable to a change in pH during thermal processing and the hydrogen ions cause the replacement...
Figure 2. Total chlorophyll (μg g⁻¹) (A), total carotenoids (μg/g) (B), total flavonoids (100 mg g⁻¹) (C), total phenols (100 g g⁻¹) (D), ascorbic acid (mg 100g⁻¹) (E) and (F) antioxidant capacity (TEAC mmol L⁻¹, % reduced DPPH) (F) in “Jurubeba” raw and subjected to different boiling times (10, 20, 30 and 40 min) and types of preservatives (oil and vinegar).
of the Mg porphyrin ring (Minguez-Mosquera et al., 1989). Other studies have also described the loss of chlorophyll in broccoli by 67.87% after cooking (Pellegrini et al., 2010).

After 10 and 40 min of boiling, jurubeba preserved in vinegar contained less chlorophyll than jurubebas preserved in oil. A loss of chlorophyll was observed, regardless of the type of preservative tested. Zhang and Hamauzu (2004) observed that cooking time was a key factor in reducing the chlorophyll content in broccoli. In the present study, no difference in chlorophyll content was observed after 20 or 30 min of boiling, regardless of the type of preservative used. Thus, it is concluded that a cooking time of 20 min, which is commonly used by preserve manufacturers, is the ideal time to maintain the green color of the jurubeba fruit.

Thermal processing did not cause an increase in the level of total carotenoids in jurubeba (Figure 2B) as compared to that in raw fruits, but the type of preservative did influence carotenoid levels; fruits preserved in oil contained higher levels of carotenoids than those preserved in vinegar. Generally, due to the high temperature in the cooking process, matrix disruption occurs, promoting the extraction of compounds in the cell and many of these compounds migrate into the cooking water. De Sá and Rodriguez-Amaya (2003) suggested that an increase in carotenoid content occurs after cooking; however, studies have demonstrated lower levels of carotenoids after thermal processing (Zhang and Hamauzu, 2004), which confirm the results of this study. After cooking, the level of total phenols increased at all cooking times as compared to that in raw fruits, regardless of the preservative used (oil or vinegar) (Figure 2C), especially when fruits were preserved in oil and boiled for 10 min. A similar increase in total phenols after heat treatment was described for eggplants (Solanum melongena) (Salerno et al., 2014).

In contrast, flavonoid levels were strongly affected by the type of preservative (Figure 2D). In vinegar, fruits showed higher levels of these polyphenols as compared to raw fruits. This increase in the content of phenols and flavonoids might be due to the high-temperature extraction/cooking time, which promoted denaturation of the fruit matrix and increased the extractability of these compounds (Turkmen et al., 2005; Zhang and Hamauzu, 2004). Cooking promotes the softening of the cell wall and other components of cells, such as vacuoles and the apoplast, releasing the phenolic compounds. Another factor that might contribute to the increase in the polyphenol content is the decomposition of phenolic compounds that are linked to fibers (cellulose and pectin) (Gökmen et al., 2009), or even the disruption of the bonds between phenols and sugars, which contributes to the increase in the polyphenol concentration (Singleton et al., 1999).

Ascorbic acid levels (Figure 2E) in fruits boiled for 20 min and preserved in oil were higher than those in raw jurubeba or those preserved in vinegar. However, this trend disappeared when cooking time increased, and fruits preserved in vinegar showed a higher ascorbic acid content.

The antioxidant capacity (Figure 2F) of jurubeba fruit, cooked and preserved in oil, increased significantly at all cooking times. The data obtained from the analysis of antioxidants in this study showed that heat treatment increased the antioxidant capacity in jurubeba.

No difference in the antioxidant capacity was observed between types of preservative after cooking for 20 min, but jurubeba fruit that was preserved in oil showed a higher antioxidant capacity after 30 and 40 min of boiling. Probably this effect is attributed to the release of antioxidant compounds from the fruit matrix as a function of the increase in temperature.

There was no difference in the polyamine content of jurubeba preserved in soybean oil between different cooking times. In addition, the jurubeba preserved in vinegar showed lower values and a decrease in putrescine was observed with an increase in the cooking time. However, spermidine and spermine levels increased together with the cooking time in jurubeba preserved in vinegar and decreased in fruits preserved in oil.

The presence of the three polyamines in the preserves was expected, because they occur naturally in fruits and vegetables (Figure 3). As compared to the levels of polyamines in raw jurubeba (putrescine, 2.01 µmols g⁻¹, spermidine 0.10 µmols g⁻¹ and spermine 1.77 µmols g⁻¹) and after thermal processing, there was an increase in the levels of putrescine and spermidine regardless of the cooking time, whereas spermine levels decreased in fruits stored in vinegar and increased in those preserved in oil.

Some studies have demonstrated that the cooking process can induce changes in the levels of polyamines. Rossetto et al. (2015) observed a reduction in the content of putrescine, spermidine and spermine in vegetables such as carrots, broccoli, cabbage and beetroot cooked in water. However, in others studies, the cooking process did not alter the levels of these amines (Eliassen et al., 2002). The cooking of jurubeba altered the levels of putrescine, spermidine and spermine. It has been reported that polyamines can be leached in boiling water, leading to a decrease in the levels of polyamines after the cooking of some vegetables. Some studies showed that a putrescine loss of about 20-25% occurred in broccoli and celery and of 40% in cauliflower and asparagus. Similarly, a 10–20% loss of spermidine occurred in broccoli and celery, and a decrease of 20–30% in cauliflower and asparagus (Ziegler et al., 1994).

Our results show that in addition to changes in the levels of polyamines induced by cooking in water, the type of preservative also has an effect. Jurubeba preserved in vinegar contained lower levels of putrescine, ranging from 2.05 to 2.21 µmol/g, where as in those preserved in oil, the levels ranged between 2.42 and 2.68.
Figure 3. Putrescine (A), spermidine(B) and spermine (μmols g⁻¹) (C) (μmols g⁻¹) in jurubeba fruits raw and subjected to different boiling times (10, 20, 30 and 40 min) and types of preservatives (oil and vinegar).
µmol/g. In vinegar preserves, lower putrescine content might indicate a better fruit quality. Biogenic amines, especially histamine, putrescine and cadaverine have been suggested to be indicators of the deterioration of some types of food such as fresh fish, meat and vegetables (Riebroy et al., 2004). These amines are important for nutrition and health. Spermidine and spermine are directly related to DNA and to cell division and their levels in jurubeba fruits cooked for 20 min were lower than at other cooking times. These results might be relevant to individuals with certain neoplasias, who possess low levels of these compounds, especially putrescine. However, spermine is important for the regulation of nitric oxide levels and absorption and can contribute to the balance of excessive production of nitric oxide. This free radical (NO) is linked to tumor progression (Til et al., 1997) and spermine has also been linked with a decrease in inflammation (Moinard et al., 2005).

Polyamines occur naturally in plants. The levels of polyamines found in this study are important in relation to the consumption of these substances, because they may be related to some heart diseases and some types of cancer. Polyamines do not cause cancer, but accelerate tumor growth. Increased levels due to the synthesis of polyamines in animal tissues and to food intake can cause increased cell growth (Mandalet al., 2015).

From the analysis of polyamines via HPLC, isoorientin, rutin and caffieic acid were identified. All polyamines were found at a lower concentration in raw Jurubeba fruits, but increased when the fruits were cooked, but the difference was not significant (Table 2). It was observed that a 10 min cooking time is sufficient to reach maximum polyphenol content. However, in other analyses, it was found that 20 min is the optimal time. A cooking time of 20 min can be used without altering the polyphenol content. Jurubeba fruits preserved in oil had a higher content of rutin, whereas those stored in vinegar had higher levels of caffieic acid. This increase in the release of flavonoids (rutin and isoorientin) and phenolic acid (caffieic acid) suggests that the antioxidant capacity within jurubeba fruits might increase or remain unchanged after cooking and when preserved either in oil or vinegar. Phenolic compounds are also water-soluble, rendering them susceptible to leaching. In our study, this effect was not observed. The cooking or the canning process did not influence the content of isoorientin, rutin or caffieic acid. Furthermore, it has been described that a decrease in these compounds occurs due to leaching into the brine rather than via oxidation (Chaovanalikit and Wrolstad, 2004).

From the obtained data, the treatment using 20 min of cooking appears to give the best results. In addition to being the most widely used treatment in homemade jurubeba preserves, this treatment did not induce changes in chlorophyll levels, an important parameter for visual analysis. In addition, some quality parameters such as pH and SS were high following this length of cooking time, as well as in both types of preservatives used, which was reflected in the ratio between SS and TA. Following cooking for 20 min, no change in the level of carotenoids were observed that could decrease the quality of the jurubeba fruits. No changes were also observed in the total phenol content, antioxidant capacity or the contents of isoorientin, rutin and caffieic acid. A further analysis confirms a cooking time of 20 min to be optimal for the lower contents of spermidine and spermine. Using this thermal treatment and vinegar as a preservative, the fruits had the lowest level of putrescine. Jurubeba preserved in oil retained more carotenoids, and a higher content of putrescine, spermidine and antioxidant capacity, and showed the highest values of pH, SS and

**Table 2.** Polyphenols content (isoorientine, rutine and acid caffieic) of raw jurubeba fruits and subjected to different boiling times (10, 20, 30 and 40 min) and types of preservatives (oil and vinegar).

<table>
<thead>
<tr>
<th>Preservative</th>
<th>Time (min)</th>
<th>Polyphenols (mg 100g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Isoorientin</td>
</tr>
<tr>
<td>Raw</td>
<td></td>
<td>21.3 ± 2.16*</td>
</tr>
<tr>
<td>10</td>
<td>81.6 ± 8.67a</td>
<td>75.38 ± 6.14a</td>
</tr>
<tr>
<td>Oil</td>
<td>20</td>
<td>80.4 ± 7.81b</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>83.7 ± 11.70a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>83.0 ± 9.91a</td>
</tr>
<tr>
<td>Vinegar</td>
<td>10</td>
<td>83.0 ± 17.04a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>85.6 ± 3.03a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>81.0 ± 0.79a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>82.2 ± 4.85a</td>
</tr>
</tbody>
</table>

*Within columns means followed by different lowercase letters are significantly different based on Tukey’s ANOVA test (P < 0.05).
SS/TA.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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Abbreviations

Cla, Chlorophyll a; Clb, chlorophyll b; Pas, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; TA, titratable acidity; SS, soluble solids.

REFERENCES


Asymbiotic seed germination and \textit{in vitro} propagation of \textit{Brasiliorchis picta}

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Seed storage method for \textit{in vitro} germination and propagation from leaves of \textit{Brasiliorchis picta} was developed. Seeds were harvested and stored at -20 and -80°C for 1, 3, 6, and 12 months and were germinated on Knudson C (KC), Murashige and Skoog (MS), half-strength MS (½ MS macro- and micro-nutrients), and woody plant medium (WPM). Seeds stored at -20°C, the recommended temperature for seed banks, had a high germination rate (76.0%) when cultivated in WPM after 12 months of storage.

WPM is the best medium for seed germination and seedling development for both harvested and stored seeds, regardless of storage time and storage temperature. Whole leaf and leaf transversal thin cell layers (TCL) from 3-month-old \textit{in vitro} grown protocorms were cultured in ½ MS supplemented with 6-benzyladenine (BA; 2.5, 5.0 and 10.0 µM) and thidiazuron (TDZ; 3.0, 6.0 and 9.0 µM) for 12 weeks. The highest frequency of regenerated protocorm-like bodies (PLBs) from explants (70.0%) occurred when whole leaves were cultured in medium containing 5.0 µM BA, whereas the best response for leaf TCL was with the basal section in medium containing 9 µM TDZ, in which PLBs developed in all regions of leaves. Plantlets were successfully acclimatized (with a survival rate of 97%) when vermiculite was used as a substrate.

Key words: Endangered species, conservation, germination rate, leaf explant, culture medium, micropropagation, Orchidaceae, thin cell layer.

INTRODUCTION

\textit{Brasiliorchis picta} (Hook.) R. B. Singer, S. Koehler & Carnevali is an epiphytic species from the family Orchidaceae (Singer et al., 2007). It is native to Southern and Southeastern Brazil including the state of Mato Grosso (Barros et al., 2015) and reach Northeastern Argentina, in Misiones (Johnson, 2001). Its value is high on the ornamental flowers market, therefore, many plants of this species have been illegally collected from wild populations, putting this species at risk of becoming endangered. The flowers are showy, fragrant and long-lasting (10 days or more), combine cream or yellowish cream colorations with purple dots or maculation (Figure...
and devoid of secretions or pluricellular trichomes that attract Meliponini bees *Trigona spinipes* (Singer and Koehler, 2004).

Storage of the orchid seeds gives us the opportunity to conserve orchids; however, there are very few studies on the longevity of orchid seeds (Suzuki et al., 2012). Many species produce enormous numbers of tiny seeds, which would allow the storage of large numbers in a reasonable small space. Orchid seeds are considered to display orthodox behavior, tolerate considerable desiccation, and storage longevity is thereby greatly increased when subjected to drying (to 5% moisture content) and freezing (-20°C), the conditions commonly utilized in orchid seed banks (Hong and Ellis, 1996; Seaton et al., 2013).

Orchid seeds do not contain endosperm and are very small, ranging in length from 0.05 to 6.0 mm (Arditti and Gani, 2000). They show quite a uniform pattern of germination and development starting with seed imbibition that leads to the rupture of the testa, release of the embryo and formation of a structure called the protocorm. Under *in situ* conditions, the germinating seed usually remains a protocorm until it is infected by a mycorrhizal fungus (Hosomi et al., 2011). Asymbiotic germination represents an ideal system for studying the nutrient composition of the medium, the source of the leaf (*in vitro/in vivo*), the part of the leaf used, and the orientation of explants (Chugh et al., 2009). Whole leaves and transversal thin cell layers (tTCLs) have been successful at micropropagation of various orchid species, such as *Dorietanopsis* hybrid (Park et al., 2002), *Aerides crispum* (Sheelavanthmath et al., 2005), *Vanda testacea* (Kaur and Bhutani, 2009), *Phalaenopsis bellina* (Khoddamzadeh et al., 2011), *Aranda×Vanda coerulae* (Gantait and Sinniah, 2012), *Renanthera* (Wu et al., 2012) and *Epidendrum secundum* (Ferreira et al., 2015).

The aim of the present investigation was to study seed storage and *in vitro* germination of *B. picta* for use in conservation, as well as to establish a protocol for mass propagation of this orchid using leaves as explants.

### MATERIALS AND METHODS

#### Plant, pollination, seed collection and storage

The plants of *B. picta* used in this study belong to the orchid collection of the Institute of Botany, São Paulo State, Brazil. Hand cross-pollination with flowers from different plants was performed and the resultant immature seed capsules, changing colouration from green to yellowish, were harvested after 3 months. The protocol of drying and storage followed that recommended by Seaton and Ramsay (2005) in their project: "Orchid Seed Storage for Sustainable Use" (OSSU). Capsules were initially washed under running tap water and a commercial detergent, and then immersed for 5 min in 70% ethanol, followed by three rinses in sterile, distilled water. The capsules were then split longitudinally with a scalpel and the seeds were placed on a Petri dish containing filter paper. Seeds were stored for seven days in a desiccator containing a saturated solution of calcium chloride (CaCl₂) at room temperature. The seeds were then transferred to tubes and placed into flasks containing silica gel. Finally, the seeds were stored in a freezer at -20 and -80°C for 1, 3, 6, and 12 months.

#### Seed sterilization, media and culture conditions

Seeds were surface-sterilized for 1 min with 70% ethanol, followed by immersion in 0.75% (v/v) sodium hypochlorite (NaOCl) plus 0.1% Tween 20® for 5 min and rinsed six times in autoclaved distilled water. Seeds were inoculated in Petri dishes (150 mm in

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**Figure 1.** *Brasiliorchis picta* flower. Collection Smidt, EC, 988. Bar=1.0 cm.
diameter and 20 mm in height) containing 40 ml of culture medium. Four basal media were tested in this study: Murashige and Skoog (MS) (Murashige and Skoog, 1962), half-strength MS (1/2 MS macro- and micro-nutrients), Knudson C (KC) (Knudson, 1946) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980). The media were supplemented with 5.6 g L$^{-1}$ agar Himedia®. 3% sucrose (w/v) and 0.1 g L$^{-1}$ inositol. The pH was adjusted to 5.8 with 0.1 N NaOH or HCl before the addition of agar. The media was autoclaved for 20 min at 120°C. The cultures were maintained in a growth room with a temperature of 26 ± 2°C/18 ± 2°C (day/night) and under a 16-h photoperiod provided by white fluorescent tubes at an intensity of 40 µmol m$^{-2}$ s$^{-1}$.

**In vitro germination and seedling development**

Seeds were inoculated under a stereomicroscope into Petri dishes containing culture medium. Four fields, each containing 100 seeds and three replicates per treatment in each Petri dish were marked. After 12 weeks, the seedling development was evaluated as one of five stages: I, rupture of the testa; II, protocorm with emerging rhizoids; III, protocorm with pointed shoot apex and rhizoids; IV, protocorm bearing one leaf; V, protocorm bearing two and more leaves and roots. The percentages of the different developmental stages were calculated by dividing the number of seeds/protocorms in each stage by the total number of seeds and protocorms (stages I to V) present in the samples. Seeds were considered germinated when the protocorm showed an initial shoot and emerging rhizoids. The germination percentage was determined after eight weeks.

**Induction and regeneration of PLBs from leaves**

Two leaves (1.0 cm in length) from 3-month old protocorms from *in vitro* germinated seeds were inoculated into Petri dishes with their abaxial face in contact with the culture medium ½ MS, supplemented with thidiazuron (TDZ; 0, 3.0, 6.0, and 9.0 µM) or 6-benzyladenine (BA; 0, 2.5, 5.0, and 10.0 µM). The experimental design was completely randomized with 3 replicates and 10 leaves per Petri dish. After 12 weeks, the percentage of explants forming protocorm-like bodies (PLBs) and the average number of regenerated PLBs per explant were determined.

**Transversal thin cell layer (tTCL)**

Leaves (1.0 cm in length) from 3-month old protocorms from *in vitro* germinated seeds were transversely sliced into six TCL sections of 1.0 mm thick (B1 and B2: basal; M1 and M2: middle section, and A1 and A2: apical section) using a sharp surgical blade. The leaf sections were cultured in Petri dishes containing 40 ml of ½ MS, supplemented with TDZ (0, 3.0, 6.0, and 9.0 µM) or BA (0, 2.5, 5.0, and 10.0 µM). The experimental design was completely randomized with three replicates and 10 sections of each region per Petri dish. After 12 weeks, the explants forming PLBs and the average numbers of regenerated PLBs per explant were determined. The values for the average number of PLBs were transformed by log x+0.5 and the percentages of explants forming PLBs were transformed by log x+0.5. Explants were transferred to ½ MS culture medium, without any growth regulator, for protocorm elongation and rooting.

**Transplanting and acclimatization**

Five-month old seedlings with two expanded leaves (5.0 cm long) from *in vitro* germination were transplanted to polystyrene trays containing: Plantmax®/vermiculite 1:1 (v/v), a mixture of Plantmax®/vermiculite 1:1 (v/v), a mixture of vermiculite/coconut powder 1:1 (v/v) and a mixture Plantmax®/vermiculite/coconut powder 1:1:1 (v/v). The seedlings were maintained in a greenhouse at room temperature and the survival percentage four months after transplanting was recorded. Seedlings were watered twice in a week. The experimental design was completely randomized, with six replicates and 10 plantlets per substrate.

**Experimental design and statistical analysis**

Experiments were performed using completely randomized designs. All data were statistically analyzed by analysis of variance (ANOVA). Means were compared by Tukey’s test at p < 0.05 using Statistica 7.0 software.

**RESULTS AND DISCUSSION**

**In vitro seed germination**

Seed germination of *B. picta* starts with swelling (Figure 2A). Then it bursts its testa (Figure 2B) and develops into a round, green form a protocorm (Figure 2C) at approximately two weeks after they were placed on culture media, regardless of the kind of media. After four weeks, rhizoids could be observed emerging from the protocorm (Figure 2D) and an apex was apparent, followed by the development of the first leaf (Figure 2E) and the second leaf. Finally, the roots thereby giving rise to a seedling after 12 weeks (Figure 2F and G). With the exception of the KC medium, all protocorms developed to the last stages. The asymbiotic seed germination of *B. picta* followed the same developmental sequence as reported for the other orchid species.

Figure 3A presents stored (-20°C) and harvested seeds germinated on all the media tested, where the seed germination rate on WPM and MS media were higher than that for the other basal media, regardless of the storage period. After 12 months of storage, the germination percentage remained high in WPM and MS media (76.0%). However, lower germination percentages occurred with the KC medium, independent of the storage period, and there was a gradual reduction in seed viability after storage (Figure 3A). Germination of *B. picta* indicate a significant interaction of storage period and culture medium for both temperatures tested (-20°C: F=2.41; G.L=12; P=0.006; -80°C: F= 4.60; G.L=12; P<0.001).

There is an increase in germination rate for seeds stored at -20°C compared to seeds stored at -80°C, independent of storage period. Higher seed germination rate also occurred in WPM and MS media for seeds stored at -80°C. For all media tested, there was a reduction of seed germination after 3 months of storage (Figure 3B).

Figure 4 illustrates the protocorm development for seeds harvested and stored at -20°C for different periods 12 weeks after *in vitro* germination in different culture media. The best response of the initial development for...
freshly harvested and stored seeds was with WPM and MS media, where the former almost always had the highest germination rate in the initial and later stages. The KC medium showed the greatest delay in seedling development. An increase in the seed storage period caused a delay in germination and protocorm development stages. Figure 4 also indicates that the storage time increased seeds germinated better on WPM than those in the other media tested.

Seed banking is an efficient method for *B. picta*
Figure 3. *Brasiliorchis picta*. Seed germination rate (%) harvested and stored at -20 and -80°C, A and B, respectively. Seeds were stored for 1, 3, 6 and 12 months and kept for 12 weeks in different culture media.

conservation considering that after 12 months of storage at -20°C, it was possible to germinate seeds with little loss of viability showing an orthodox behavior. The temperature of -20°C was recommended for orchid seed banks by Seaton et al. (2013). According to Machado Neto and Custodio (2005), seeds stored at -20°C and 5% moisture content can avoid attack of pathogens and activation of metabolic processes that would age seeds too fast, leading to rapid viability loss. The freshly harvested seeds had the highest germination rate, followed by those stored at –20°C, with a gradual reduction as storage period increased. Hay et al. (2010), who tested seed storage temperatures range -196 to +23°C also found that the ideal temperature to maintain the viability of several orchid species was -20°C including *Diuris laxiflora*.

Seed germination and seedling development of *B. picta* were affected by the type of medium, WPM with fresh harvested seeds and those stored at -20 and -80°C gave the best results: about 80, 76 and 69%, respectively. Plantlets cultured in WPM medium were more vigorous and showed no evidence of necrosis, as was also observed for plantlets developed with MS medium after eight weeks of culture. Suzuki et al. (2012) also reported that the germination and protocorm/seedling development are greatly influenced by different culture media and among different species the results vary significantly. They recommended the use of MS and KC medium for *Hoffmannseggella cinnabarina*. In the present study, seeds of *B. picta* cultured in MS medium initially showed good results, whereas the KC medium produced slow development of the protocorms and they were light-yellowish in color, like in research on *Cymbidium aloifolium* by Pradhan et al. (2013). Johnson and Kane (2007) also reported that protocorm development of *Vanda* hybrid seeds germinated on ½ MS was consistently more advanced than development with KC, and suggested that this may be linked to a low ammonium to nitrate ratio. MS is highly enriched with macro- and micro-elements, ½ MS and WPM media contain a low amount of macro- and micro-elements, but are enriched with different vitamins, whereas KC contains a low amount of macro- and micro-nutrients and lacks vitamins (Hossain et al., 2010). Thus, the nutrient regime for orchid culture is species specific and no single culture medium is universally applicable to all orchid species (Pradhan et al., 2013).

**Induction and regeneration from whole leaves**

After five weeks of culture, induction of PLBs was visible with white globular structures observed in the basal region of the leaves (Figure 5A). After eight weeks, greenish protuberances increased size and were observed on the surface of leaves. Approximately 30% of the explants produced 2.0 PLBs per responsive explant after 12 weeks of culturing on growth regulator-free WPM (Table 1). A similar response was observed for an orchid hybrid (*Aranda*×*V. coerulea*), in which PLB formation was seen even with growth regulator-free medium, but the frequency was low with very few PLBs. In addition, the time taken for induction of PLBs was much longer as compared to the media with growth regulator. One logical explanation for the occurrence of PLBs in the control medium is endogenous cytokinin (Gantait and Sinniah, 2012).

Analysis of variance of the explants forming PLBs and the average number of PLBs per explant indicated that there were no statistical differences between treatments
Cytokinin type and concentration did not influence regeneration of PLBs or the average number of shoots, but the explants cultured in half-strength MS medium containing 5.0 µM BA produced the highest percentage of new PLBs (70%) with 13.2 PLBs per explant (Table 1 and Figure 5B). Lesser results were obtained for leaf explants of *V. testacea*, for which the best regeneration of PLBs (48.7%) was obtained in Mitra medium containing a similar concentration of BA (4.4 µM) (Kaur and Bhutani, 2009), as used in this study. BA concentration also influenced the response of explants of *E. secundum*; leaves cultured in a medium supplemented with 1.0 to 5.0 µM BA regenerated more PLBs than the control (Ferreira et al., 2015). The addition of 5.0 µM of BA to WPM medium also induced high regeneration rate (70.0%), but the average number of PLBs per responsive explant was lower (2.0) (Ferreira et al., 2015). Similarly, for leaf explants of *Dendrobium* hybrids, BA was efficient...
Figure 5. Plant regeneration and protocorm-like bodies (PLBs) developing from leaf explants of *Brasiliorchis picta* cultured on half-strength MS medium. A-B: Whole leaf with initiation of a PLB after five weeks and PLBs developing in medium supplemented with 5 µM BA after eight weeks of culture, respectively. C: PLBs cultured in medium with 6 µM of thidiazuron (TDZ) showing the first leaf primordial. D: PLBs developing from basal leaf section with medium supplemented with 3 µM. E-F: Formation of multiple PLBs along the basal leaf section in medium supplemented with 9 µM of TDZ after 8 and 10 weeks of culture, respectively (Scale bars = 1000 µm).

in MS medium, but the concentration used was ten times greater (44.4 µM) than that used in our study. This high concentration induced 60% shoot regeneration and 7.5 shoots per leaf explant (Martin and Madassery, 2006), which are lower than that for *B. picta* leaves. Since there were no significant differences in ratio of PLBs regeneration and average number of PLBs, it can be concluded that there is great variability in PLB formation. Therefore, cytokinin and/or culture time must be optimized and that future studies need to include more repetitions.

**Transversal thin cell layer (tTCL) of leaves**

Figure 5 presents the formation of PLBs after four weeks of culture, most frequently in the basal sections (B1) (Figure 5C to E) followed by B2 sections (Figure 5F), but the response of the middle region was poor (Table 2). PLBs were well differentiated on explants by the end of eight weeks and the first leaf primordia were observed after 12 weeks (Figure 5F). PLB formation was also observed on medium without growth regulator, but the frequency was low (26.7%) with very few PLBs (1.6) and only in the basal region (Figure 5). Among the cytokinins tested, the development of PLBs was more efficient in the presence of TDZ (Figure 5C to F), as compared to BA (Figure 5A and B). The best response was recorded for the medium containing 9 µM TDZ, in which PLBs developed in all regions of the leaves (Table 2, Figures 5E, 5F, and Figure 6). These responses demonstrate that the addition of cytokinin is necessary for organogenesis from leaf TCL and the medium supplemented with BA caused necrosis of PLBs, after 12 weeks (Table 2). The
Table 1. *Brasiliorchis picta*. Regeneration of protocorm–like bodies (PLBs) and the average number of PLBs per explant from leaves after 12 weeks of culture in Murashige and Skoog medium at half strength of salts (½ MS) supplemented with cytokinins.

<table>
<thead>
<tr>
<th>Cytokinins (µM)</th>
<th>Regeneration of PLBs (%)</th>
<th>The average number of PLBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.0 (±0.1)</td>
<td>2.0 (±0.5)</td>
</tr>
<tr>
<td>BA 2.5</td>
<td>40.0 (±0.3)</td>
<td>2.1 (±1.0)</td>
</tr>
<tr>
<td>BA 5.0</td>
<td>70.0 (±0.3)</td>
<td>13.2 (±6.6)</td>
</tr>
<tr>
<td>BA 10.0</td>
<td>60.0 (±0.2)</td>
<td>7.1 (±3.0)</td>
</tr>
<tr>
<td>TDZ 3.0</td>
<td>53.3 (±0.2)</td>
<td>8.2 (±4.6)</td>
</tr>
<tr>
<td>TDZ 6.0</td>
<td>53.3 (±0.3)</td>
<td>11.4 (±13.2)</td>
</tr>
<tr>
<td>TDZ 9.0</td>
<td>60.0 (±0.3)</td>
<td>9.0 (±2.0)</td>
</tr>
<tr>
<td>Mean</td>
<td>52.4</td>
<td>7.6</td>
</tr>
</tbody>
</table>

BA: 6-benzyladenine; TDZ: thidiazuron.

Table 2. *Brasiliorchis picta*. Effect of leaf tTCL regions (B1-B2 – basal region, M1-M2 – middle region and A1-A2 apical region) and cytokinins on protocorm-like-bodies (PLBs) regeneration after 12 weeks of culture in ½ MS medium.

<table>
<thead>
<tr>
<th>Cytokinins (µM)</th>
<th>Regeneration of PLBs (%)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B1</td>
<td>B2</td>
</tr>
<tr>
<td>Control</td>
<td>26.7Ab</td>
<td>10.0bcA</td>
</tr>
<tr>
<td>BA 2.5</td>
<td>40.0Ab</td>
<td>13.3Ab</td>
</tr>
<tr>
<td>BA 5.0</td>
<td>43.3Ab</td>
<td>23.3abcA</td>
</tr>
<tr>
<td>BA 10.0</td>
<td>36.7Ab</td>
<td>30.0abA</td>
</tr>
<tr>
<td>TDZ 3.0</td>
<td>43.3Ab</td>
<td>30.0abA</td>
</tr>
<tr>
<td>TDZ 6.0</td>
<td>46.7Ab</td>
<td>36.7abB</td>
</tr>
<tr>
<td>TDZ 9.0</td>
<td>46.7Ab</td>
<td>40.0abA</td>
</tr>
<tr>
<td>Mean</td>
<td>40.5</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Means within a column followed by the same lower case letter and means within a row followed by the same upper case letter do not differ significantly according to Tukey’s test (P ≤ 0.05). ½ MS Murashige and Skoog medium at half-strength of salts. BA: 6-benzyladenine; TDZ: thidiazuron.

Results indicated a significant effect of the type of TCL and type of cytokinin on the formation of PLBs of *B. picta* (F=3.31; G.L=30; P<0.001).

The addition of cytokinin to culture medium was also necessary to produce PLBs from tTCLs of leaves of *B. picta*. While BA was more efficient at producing PLBs with whole leaves, TDZ provided better results for tTCL. TDZ was used alone for *P. bellina* and also formed PLBs directly from the surfaces of the leaf segments (Khoddamzadeh et al., 2011). The efficiency of TDZ for PLB induction was also reported for basal leaf sections of *Renanthera* (Wu et al., 2012). Foliar sections (1.0 mm thickness) of *Dorietanopsis* hybrid, cultivated in ½ MS and supplemented with 9 µM of TDZ, produced the highest ratio of PLB formation (72.3%) and the greatest number of PLBs per explant (18.0) (Park et al., 2002). In the present study, in the same conditions, the ratio of PLB regeneration was lower (46.7%), but the average number of PLBs was higher (23.1). If the number of PLBs formed in all the regions of the leaves in culture medium containing 9 µM of TDZ was added, 43.6 PLBs can be produced, indicating that the tTCL technique is promising for *B. picta*.

In the present study, young whole leaf and leaf segments of *B. picta* developed PLBs through direct organogenesis on media supplemented with cytokinins. The beginning of PLB formation occurred initially in the basal region for all explants after 4 weeks of culture. The middle leaf region was not responsive and the apical region produced a low frequency of PLB regeneration.

Like in others monocots, orchid leaf base is meristematic and produces plantlets when excised and cultured (Seeni and Latha, 1992). Similar response was also observed for *V. coerulea*, where the adventive meristematic cells spread all over the surface of the leaf base responded to favorable culture conditions initially by random mitotic divisions and then organization into PLBs (Seeni and Latha, 2000). Kaur and Buthani (2009) also observed that only basal sections of *V. testacea* responded to cultivation.
explained that the high potential of regenerating PLBs from leaf sections of the *Aranda×V. coerulea* hybrid can be attributed to the injury response of the cut surface of leaves activating quiescent cells and initiating cell multiplication.

**Transplanting and acclimatization**

Plantlets grew vigorously during 12 weeks of cultivation in a greenhouse and they showed higher survival rate after transplanting with vermiculite or a mixture of vermiculite, Plantmax® and coconut powder (1:1:1). The plantlets maintained in a substrate of only Plantmax® had higher mortality rate (Table 3).

Plantlets of *B. picta* were successfully acclimatized in a greenhouse using vermiculite as a substrate (97% survival rate after 12 weeks). They grew fast and formed new leaves. Faria et al. (2001) analyzed the growth of *Oncidium baueri* and *B. picta* plants in a greenhouse and showed that vermiculite is an excellent substitute substrate for xaxim (*Dicksonia sellowiana*), as observed in our study. However, they recommended a mixture of vermiculite and charcoal or carbonized rice husk substrates for *B. picta*. Similar response was obtained for plantlets of *Brasilidium forbesii* with 100% of survival rate in the greenhouse using vermiculite as a substrate (Gomes et al., 2015). The ideal substrate for ornamental plant cultivation should be available in great quantity, be easy to handle and cheap (Faria et al., 2001), as observed for vermiculite.

Based on the results obtained in our study, we recommend the use of WPM medium for seed germination and seedling development of *B. picta*. Seeds stored at -20°C have a high germination rate, so it remains necessary to evaluate them for periods greater than 12 months. Whole leaves and leaf transversal thin cell layers from 3-month-old *in vitro* grown protocorms...
can be used for PLBs regeneration when 1/2 MS is supplemented with 5 µM BA or 9 µM TDZ, respectively. Vermiculite can be used as substrate for transplanting and acclimatization of plantlets in a greenhouse.

Conclusion

An efficient method for seed conservation, for the in vitro germination and for the direct regeneration of a large number of plantlets from leaves of B. picta has been described. This report could be applied to mass-scale propagation as well as ex situ conservation for floriculture of this important, but threatened orchid species.

Conflict of interests

The authors have not declared any conflict of interests.

Abbreviations

BA, 6-Benzyladenine; KC, Knudson C; MS, Murashige and Skoog; MS or MS/2, half-strength MS macro- and micro-nutrients; PLBs, protocorm-like bodies; TDZ, thidiazuron; tTCL, transversal thin cell layer; WPM, woody plant medium.

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REFERENCES


Possible oxidative effects of isotretinoin and modulatory effects of vitamins A and C in Saccharomyces cerevisiae

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Isotretinoin (ITN), chemically known as 13-cis-retinoic acid, is a part of the broad group of compounds related to vitamin A. It is particularly employed in the treatment of nodular cystic acne and as an inhibitor of proliferation of neoplastic cells, by exerting a regulatory effect on the cell differentiation. This study aimed at investigating the possible oxidative effects of ITN and modulatory effects of vitamins A and C in mutant and non-mutant Saccharomyces cerevisiae strains. In addition, to reconfirm the oxidative effects, five in vitro antioxidant assays were also prepared taking the alpha-tocopherol analogue, trolox as a standard. In vivo study conducted on S. cerevisiae cells was carried out with ITN 20 µg/ml taking hydrogen peroxide (H₂O₂) as stressor (STR), whereas ITN 5 to 50 µg/ml was considered for in vitro assays taking similar dose of trolox (TRO). Results show ITN to have oxidative effect in both in vitro and in vivo tests. In conclusion, ITN produced oxidative effects and there may be an hypervitaminosis effect with vitamins A and C, thus insinuation to genetic material.

Key words: Assay, isotretinoin, vitamin A, vitamin C, oxidative stress.

INTRODUCTION

Isotretinoin (ITN) is a chemical compound known as 13-cis-retinoic acid, marketed under the name Roaccutane; a synthetic isomer of tretinoin (TN) administered systemically. It is a drug under the class of retinoids (RTD), which according to Brito et al. (2010) is related to vitamin A (Vit A) having a similar chemical structure being
considered the only natural RTD (first generation) undergoing clinical application through systemic therapy for acne treatment.

The RTD are involved in the proliferation and differentiation of various types of cells during fetal development and throughout the life as well as in the activation of retinoid-receptor complex. On the other hand, the activation of the complex can block the action of other transcription factors as AP1 whose expression appears to be exacerbated in various hyperproliferative and inflammatory conditions (Diniz et al., 2002).

The pharmacokinetic characteristics of ITN are analogous to Vit A, which after oral administration results in high plasma concentrations of ITN at the first 2 to 4 h. As a result, an oxidation process occurs and by cytochrome P450 enzymes in the stomach wall gives rise to 4-oxo-metabolites of ITN, TN and 4-oxo-tretinoin (OTN). OTN is evident to accumulate and increase the concentration in systemic circulation by consecutive administrations (Cajueiro et al., 2014).

Vitamins are organic substances essential to normal metabolism of living beings, they act as cofactors for enzymatic reactions, and their deficiency leads to malfunctioning of the organism (avitaminosis). However, the excess of vitamins also creates some hypervitaminosis related problems. Vit A, and more specifically, the retinoic acid (RA), is shown to maintain differentiation of keratinocytes (immature skin cells) in hypervitaminosis related problems. Vit A, and more specifically, the retinoic acid (RA), is shown to maintain normal skin health by switching on genes and differentiation of keratinocytes (immature skin cells) in mature epidermal cells (Fuchs, 1981).

According to Pires (2008), ascorbic acid (AA), also known as vitamin C (Vit C), or ascorbate, is a lactone (C6H12O6) whose molecular weight is 176.13 daltons, an essential ingredient in the metabolism of living cells with numerous physiological properties. The conversion of Vit C as dehydroascorbic acid normally occurs inside the body reversibly. This transformation capacity acts as an oxidoreductro system and is able to transport hydrogen in the breath processes at the cellular level. Azulay et al. (2003) claim that Vit C can acts as a pro-oxidant, promoting the formation of reactive oxygen species (ROS) such as H2O2, which compromise cell viability.

The main target of ROS include DNA, lipids, proteins and sugars, and their attacking order preferentially depends on numerous factors, such as the site of generation, types of macromolecules to be oxidized and the availability of metal ions metal associated to the biomolecules. However, the oxidized lipids, proteins and sugars can be removed via degradation which is not common phenomena for the DNA; since the molecule is responsible for all genetic information of all cells of a living organism (Berra et al., 2006), thus the oxidation to DNA may be a detrimental effect to all living cells.

Induction of oxidative damage to DNA bases occurs from its reaction with ROS. Lesions can occur due to direct oxidation of the nucleic acids or often, lead to the formation of breaks on one of the DNA strands (single breaks - SSB "single strand break") or single failures in approximately symmetrical positions on both strands of the DNA (double breaks - DSB "double strand break"). In addition, simple breaks can generate double breaks during cell replication (Valadares et al., 2012).

Taking into account that ITN as a drug with exaggerated doses of Vit A analogues may induce damage to the genetic material, thus the present study was undertaken to evaluate the net oxidative effects of ITN and the modulatory effects of Vit A and C in one proficient, three single mutants and two double mutants Saccharomyces cerevisiae strains. And to reconfirm the oxidative effect, some in vitro, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS**, hydroxyl radical •OH, nitric oxide (NO) and reduction potential (RP) antioxidant assays were also prepared.

MATERIALS AND METHODS

Research type

To access the interaction of ITN with Vit A and C, this study was conducted in the Laboratory of Pharmacology and Genetics (LAPGENIC) at Federal University of Piauí (UFPI), Brazil.

Sources of reagents/chemicals and preparation for test concentrations

ITN was purchased from the Popular Pharmacies Teresina-PI, through a prescription given by a dermatologist. ITN at a concentration range of 5 to 50 µg/ml for in vitro while for in vivo antioxidative assay 20 µg/ml were used. Vit A and C were used at concentration of 17 µg/ml. All the other necessary reagents/chemicals used in this study were collected from Sigma-Aldrich, St. Louis, MO; USA.

In vivo assay

Used S. cerevisiae strains

Table 1 contains the proficient (SodWT), single deficient (Sod1Δ, Sod2Δ and Cat 1Δ) and double deficient (Sod1ΔSod2Δ and Sod1Δ/Cat 1Δ) of S. cerevisiae test strains.

Central disk test in yeast S. cerevisiae (in vivo)

This test was performed according to the aerobic metabolism pathway earlier described by Fragoso et al. (2008). Briefly, previously sub-cultured strains were linearly swabbed to the sterile Yeasts Extract Peptone Dextrose Broth (YEFD) media (0.5% yeast extract, 2% peptone, 2% dextrose and 2% bacteriological agar). Then 0.01 ml of test stressor (H2O2, STR) /STR/netagive control (NC) (specified concentrations) was applied on sterile paper disks and was treated accordingly, such as for alone (NC/ITN/STR) and co-treatment groups (ITN/Vit A/Vit C). The NC and STR groups were treated with sterile vehicle (0.9% NaCl) and 50 mM STR, respectively. Treatments were done immediately after swabbing the organisms in petri-dishes. Linearly organisms swabed are as shown in Figure 1. Dishes were then inverted (180°), kept into an incubator maintaining temperature 35±1°C for 72 h, followed by the
Table 1. Description of *Saccharomyces cerevisiae* strains used in the study (Oliveira et al., 2014).

<table>
<thead>
<tr>
<th>Description</th>
<th>Genotypes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG103 (SodWT)</td>
<td>MAT α leu2 3, 112 his3 Δ 1, trp1 - 289 ura-3 52</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
<tr>
<td>EG118 (Sod1Δ)</td>
<td>EG103 except sod1::URA3</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
<tr>
<td>EG110 (Sod2Δ)</td>
<td>EG103, except sod2:: TRP1</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
<tr>
<td>EG133 (Sod1ΔSod2Δ)</td>
<td>EG103 except sod1::URA3 sod2::TRP1</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
<tr>
<td>EG 223 (Cat1Δ)</td>
<td>EG103 except cat1::TRP1</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
<tr>
<td>EG (Sod1ΔCat1Δ)</td>
<td>EG103 except sod1::URA3cat1::TRP1</td>
<td>Edith Gralla E, L Angeles</td>
</tr>
</tbody>
</table>

**Figure 1.** Test strains of *S. cerevisiae* positioning in the Petri dish.

Measurement of inhibition zones in millimeters (mm) with a range from 0 mm (full growth) to 40 mm (no growth); these values being the size of the petri-dishes procured. All the treatments were performed in duplicate.

Percentage oxidative activity was calculated by using the following formula:

\[
\text{% oxidant} = \frac{[\text{STR-test group}] - \text{STR}}{\text{STR}} \times 100.
\]

**In vitro reconfirmation assays**

**DPPH radical scavenging test**

For the DPPH radical scavenging test, methodology described by Silva et al. (2005) with minor modifications was used. ITN and standard TRO were used at concentrations of 5, 10, 25 and 50 µg/ml. The antioxidant evaluation was performed calculating the percentage of scavenging of DPPH radicals by the following equation:

\[
\text{% scavenged DPPH radical} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}}
\]

Where \(A_{\text{control}}\) is the initial absorbance of DPPH in ethanol (absolute) solution, and \(A_{\text{sample}}\) is the absorbance of the reaction mixture containing ITN/TRO. Absorbance was taken at 517 nm. The half-minimal effective inhibitory concentration (EC₅₀) of the ITN/TRO was also determined. A NC was considered without sample.

**ABTS radical scavenging test**

This test was performed by the method earlier described by Re et al. (1999). Initially, the ABTS cationic radical is formed from the reaction of 5 ml of a 7 mM ABTS in 0.088 ml of a 2.45 mM solution of potassium persulfate (K₂S₂O₈), and incubated at room temperature in the absence of light for 16 h, then followed by dilution in ethanol to attain a solution of absorbance 0.70 ± 0.05 at 734 nm. Now, in a dark environment at room temperature, 0.040 ml of specified concentrations of ITN/TRO was transferred to vials with
containing 6 mM phosphate buffer (pH 7.4). The reaction µg/ml) was added to the Fenton reaction mixture.

● phosphate buffer (pH 7.4). Once generated, nitric oxide decomposition of sodium nitroprusside (SNP) in 20 mM.

This test was produced from the spontaneous minor modifications was used for the hydroxyl radical ('\( \cdot \)OH) scavenging test. Briefly, ITN/TRO (5, 10, 25 and 50 µg/ml) was added to the Fenton reaction mixture containing 6 mM phosphate buffer (pH 7.4). The reaction was carried out for 15 min at room temperature and stationed by the addition of phosphoric acid at 4% (v/v) and followed by addition of 1% thiobarbituric acid (TBA) (w/v in 50 mM NaOH). The reaction mixture was then heated for 1 h at 37°C and subsequently was cooled to room temperature. Finally, the absorbance was taken at 532 nm and percentage of radical scavenged as well as EC\(_{50}\) were calculated accordingly.

\[ \text{Reducing potential (\%)} = \left( \frac{A_{bt} - A_{bs}}{A_{bt}} \right) \times 100 \]

where \( A_{bt} \) is the absorbance of test sample and \( A_{bs} \) is the absorbance of blank sample.

\[ \Delta \text{Test strains} \]

\[ \begin{array}{cccccc}
\text{Group} & \text{SodWT} & \text{Sod1Δ} & \text{Sod2Δ} & \text{Sod1Sod2Δ} & \text{Cat1Δ} & \text{Cat1Sod1Δ} \\
\text{NC} & 0.90±0.14 & 0.25±0.3 & 1.50±0.70 & 0.50±0.70 & 0.05±0.07 & 0.50±0.70 \\
\text{STR} & 11.0 ± 1.4 & 15.0±2.82 & 16.50±2.12 & 14.0±2.82 & 18.5±0.70 & 16.5±2.12 \\
\text{ITN (20 \( \mu \)g/ml)} & 3.05±0.2\text{a} & 3.50±0.70\text{a} & 3.75±0.35\text{a} & 3.65±0.49\text{a} & 3.70±0.14\text{a} & 3.70±0.14 \\
\text{ITN (20 \( \mu \)g/ml) + Vit A (17 \( \mu \)g/ml)} & 3.05±0.21\text{a} & 3.80±0.28\text{a} & 3.95±0.07\text{a} & 3.4 ± 0.28\text{b} & 3.75±0.07\text{a} & 3.20±0.28 \\
\text{ITN (20 \( \mu \)g/ml) + Vit C (17 \( \mu \)g/ml)} & 1.70±0.14\text{a} & 1.05±1.06\text{a} & 2.95±0.21\text{a} & 1.55±0.63\text{a} & 3.45±0.21\text{a} & 2.70±2.28 \\
\text{ITN (20 \( \mu \)g/ml) + Vit A (17 \( \mu \)g/ml) + Vit C (17 \( \mu \)g/ml)} & 1.15±0.21\text{a} & 3.60±0.14\text{a} & 3.55±0.35\text{a} & 0.85±0.21\text{a} & 4.00±0.0\text{a} & 2.10±0.14 \\
\end{array} \]

NC: Saline (0.9% NaCl); STR: stressor (\( \text{H}_2\text{O}_2 \)); ITN: isotretinoin; Vit A: vitamin A; Vit C: vitamin C; one-way ANOVA followed by Tukey’s multiple comparison test with significance \( p<0.0001 \), and \( p>0.0001 \) compared with NC.

RESULTS AND DISCUSSION

Oxidative effect of ITN in \textit{S. cerevisiae}

In this \textit{in vivo} study, ITN produced significant oxidative damage to all strains tested as compared to NC (Table 2). Since ITN is showing no statistical differences with STR group, thus suggesting it should be a ROS inducer to the \textit{S. cerevisiae} strains.

ITN is a Vit A derivative that acts by binding to retinoid receptors, which participate in the growth and differentiation of cells and that is used in severe forms of acne (Brito et al., 2010). Besides this ITN is one of the most widely prescribed drugs which reduce the size of sebaceous glands and their secretion as well as the number of bacteria in both channels and the skin surface. This is thought to be not only a result of the reduction of sebum, the nutrient source for bacteria, but also a reduction of the inflammation by inhibiting chemotactic responses of neutrophils and monocytes (Nasser et al., 2011).
Lupulescu (1993) and Rutkowski (2012) emphasize that Vit A is a micronutrient, and due to its fat solubility, it is easily transformed into the human body in RA (the active form), the major forms may be mentioned as all trans-RA (ATRA, the most important) and 9-cis RA (9-cis RA); these are important for the cell growth and differentiation. However, ITN in large dose may produce hypervitaminosis which may develop trouble to the normal functions of the body due to over-production of free radicals (RLs). However, ITN is evidenced to design oxidative stress in vivo tests by using eukaryotic cells (Duester, 2008). According to Vellosa et al. (2013), oxidative stress is regarded as a metabolic condition where an imbalance occurs in pro- and antioxidant systems or body defense system; these conditions can mediate cell damage through oxidation of bio-macromolecules, such as, lipids, proteins and DNA.

The metabolism of retinoids causes the rearrangement of three structural parts of their skeleton, justifying the existence of multiple analogues with potentially different biological effects (Gundersen and Blomhoff, 2001); otherwise, due to the oxidation process, the metabolism of ITN by cytochrome P450 in the stomach wall giving rise to 4-oxo-ITN metabolites, TN and 4-oxo-TN, where the main metabolite is 4-oxo-ITN, which eventually accumulates in the blood from consecutive administrations.

Co-treatment result of ITN with Vit A in S. cerevisiae

ITN at a dose of 20 µg/ml with 17 µg/ml of Vit A also showed significant oxidative damage (Table 2). There may be hypervitaminosis effect which is evident to induce oxidative stress in S. cerevisiae (Duester, 2008). According to Rodriguez et al. (2001), the wild proficient strain (SodWT) is produced for the defence of the cytoplasm and mitochondria. The enzyme responsible for the detoxification of STR is catalase (CAT); hence a decrease in the activity of this enzyme and an increase in the production of STR, can result in selective cytotoxicity of Vit C (Silva et al., 2012).

Ferrini et al. (2001) stated that the chemical tests for antioxidant activity are faster and simpler carry out. However, they are no representative cellular conditions in human. Microbial in vivo tests by using eukaryotic cells are most popular, and in this context, yeasts have been proven as suitable microorganisms for the determination of the antioxidant capacity of different compounds due to their rapidity, reproducibility and correlation with respect to humans.

Co-treatment results with Vit C in S. cerevisiae

A shown in Table 2, it is clear that ITN 20 µg/ml co-treated with 17 µg/ml of Vit C improved the survival of the tested S. cerevisiae strains in comparison to the Vit A co-treated ITN group. As Vit C is a well known antioxidant, there may be the effect of it. However, the damage was not significantly inhibited by this co-treatment. Vit C positively affects the immune system, minimizes the risk of inflammation, thereby the antioxidant activity of this vitamin has been intertwined with the protection of cell function in the appearance of free radicals by oxidative stress, thus the controlling of aging, inflammatory damage, and cancer (Nasser et al., 2010).

In addition, vitamins are the essential trace substances to the human body, but the recommended supplementation should be evaluated specifically for each case, as there are many organic and inorganic components in cells that can modulate the activity by them more specifically using Vit A and C (Lupulescu, 1993; Santos, 2012).

Co-treatment of ITN with Vit A and C in S. cerevisiae

In this occasion, again the oxidation effects come back. There may be a net effect due to the incorporation of Vit A and the induction hypervitaminosis. Going through data presentation (Table 3), it is clear that the oxidative effect was more prominent to the single mutants Sod1Δ, Sod2Δ and Cat1Δ. CAT is an antioxidant enzyme that is normally produced in all living organisms; it has an important role in protecting the body acting the defense against STR, protecting synergistically the cells. Furthermore, it has an action to retard or inhibit oxidation, thus acting at different levels of oxidative sequence by adapting mechanisms to oxidative stress (Silva et al., 2012).

However, Catania et al., (2009) reported that regular consumption of foods rich in Vit A and C can decrease the incidence of rectal and colon cancers. Carotene, which is the most important precursor of Vit A, is widely distributed in food and possess antioxidant capacity. It is absent in S. cerevisiae strains and is used in the concomitant therapy by ITN, Vit A and C; although eukaryotic cells are similar and have the same defenses similar to that of the cells in humans.

DPPH scavenging assay

DPPH radical scavenging assay is the most commonly performed test method in which antioxidant activity can be determined by monitoring the decrease in absorbance of the test sample. Anti-DPPH radical scavenging activity is tested here. Trolox, (+)-catechin, ethyl gallate, ascorbic acid and α-tocopherol are generally taken as standards (Antolovich et al., 2002). In our test, ITN did not show significant antioxidant activity in all tested concentrations as compared to the TRO (standard). The EC50 calculated for ITN was 21.23 ± 1.14 µg/ml, while for TRO it was
Table 3. In vitro reconfirmation assays adopting some antioxidant test methods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DPPH test (% radical scavenging)</th>
<th>ABTS test (% radical scavenging)</th>
<th>OH test (% radical scavenging)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITN</td>
<td>TRO</td>
<td>ITN</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>6.04±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.90±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.21±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>1.37±0.01</td>
<td>7.88±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.68±0.01</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>4.73±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.69±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.97±0.01</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>3.97±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.73±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.41±0.01</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>21.23±1.14</td>
<td>17.25±8.46</td>
<td>17.22±1.18</td>
</tr>
<tr>
<td>Cl (µg/ml)</td>
<td>3.49-141.9</td>
<td>7.91-37.66</td>
<td>2.97±0.01</td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.74</td>
<td>0.94</td>
<td>0.71</td>
</tr>
<tr>
<td>CTL</td>
<td>1.19±0.05</td>
<td>1.29±0.03</td>
<td>2.11±0.02</td>
</tr>
</tbody>
</table>

NO test (% radical scavenging) | RP test (% reduction capability)
| ITN | TRO | ITN | TRO |
| 5 µg/ml | 9.48±0.01<sup>a</sup> | 9.79±0.01<sup>a</sup> | 6.16±0.01<sup>a</sup> | 16.45±0.01<sup>a</sup> |
| 10 µg/ml | 6.14±0.01<sup>a</sup> | 15.16±0.01<sup>a</sup> | 5.82±0.01<sup>a</sup> | 36.25±1.42 |
| 25 µg/ml | 6.68±0.01<sup>a</sup> | 22.02±0.02<sup>a</sup> | 2.99±0.01<sup>a</sup> | 36.25±1.42 |
| 50 µg/ml | 3.52±0.02 | 25.23±0.01<sup>a</sup> | 2.63±0.01<sup>a</sup> | 36.25±1.42 |
| EC<sub>50</sub> (µg/ml) | 36.25±1.42 | 7.10±4.01 | 33.34±1.07 | 7.10±4.01 |
| Cl (µg/ml) | 6.26-209.9 | 5.05-9.99 | 12.26-90.69 | 12.26-90.69 |
| R<sup>2</sup> | 0.76 | 0.98 | 0.89 | 0.94 |
| CTL | 1.33±0.01 | 1.33±0.04 |

ITN: isotretinoin; TRO: torlox; CTL: control (chloroform); EC<sub>50</sub>: half minimal effective concentration; CI: confidence interval; R<sup>2</sup>: coefficient of determination; p<0.05 with 95% confidence interval where *compared to CTL, †for 5 µg/ml, ‡for 10 µg/ml, §for 25 µg/ml, ¶for ITN and TRO similar dose (one-way ANOVA Tukey test); values are the mean±SEM (n=5).

17.25 ± 8.46 µg/ml. The increase in doses also reduces the capacity of radical scavanged (Table 3), there may be an oxidative-like activity by large doses of ITN.

**ABTS**<sup>**</sup> scavenging assay

ABTS assay is the other familiar method for antioxidant test, where the scavenging activity of ABTS**<sup>**</sup> radicals is done. It is a peroxidase substrate upon oxidation in the presence of H<sub>2</sub>O<sub>2</sub> that generates a metastable radical cation (Antolovich et al., 2002). As shown in Table 3, it is clear that increased concentration reduced radical (ABTS**<sup>**</sup>) scavenging activity by the ITN, which is ever opposite than the TRO. The EC<sub>50</sub> calculated for ITN was 17.22 ± 1.18 µg/ml and for TRO was 16.45 ± 11.93 µg/ml, respectively.

**OH**<sup>**</sup> scavenging assay

Hydroxyl free radical (**OH) generally reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes cellular destruction (Alam et al., 2013). Hydroxyl anionic radical measurement is done similarly to the DPPH and ABTS assays, where **OH is produced from the H<sub>2</sub>O<sub>2</sub> and is scavenged by the sample tested. The ITN did not show antioxidant activity by reducing the **OH in all concentrations tested, compared to TRO. The EC<sub>50</sub> calculated for ITN was 44.77 ± 0.95 µg/ml, while for the TRO it was 13.54 ± 7.70 µg/ml (Table 3).

**NO**<sup>●</sup> scavenging assay

Sodium nitroprusside in NO**<sup>●</sup> scavenging test is used as nitric NO radical source. In aerobic conditions, NO reacts with oxygen to produce stable nitrate (NO<sub>3</sub>**<sup>−</sup>) and nitrite (NO<sub>2</sub>**<sup>−</sup>) radicals, quantities of which can be determined using Griess reagent (Marcocci et al., 1994). In the NO kidnapping test, ITN by 5 µg/ml exhibited similar activity like that of the TRO, but it was again discontinued from the 10 to 50 µg/ml as augmented doses caused reduction of inhibitory activity. The values of EC<sub>50</sub> for ITN and TRO were calculated as 36.25 ± 1.42 and 7.10 ± 4.01 µg/ml, respectively (Table 3).

**RP test**

One of the rapid and sensitive antioxidant test method is
the RP assay, in which absorbance is increased by the formation of color complex with potassium ferricyanide, trichloro acetic acid and ferric chloride reaction. An increase in the absorbance indicates antioxidant activity of the test sample (Jayaprakash et al., 2001). There is also a similar result like that of the ABTS+ assay, as increased doses caused reduction of the antioxidant potential of ITN. However, TRO was found to have potential reducing capability in this occasion with an EC50 of 3.55±7.40 μg/ml and ITN of 33.34±1.07 μg/ml (Table 3).

Although the living system is co-existed with free radicals by developing diverse mechanisms for adapting them to advantageous physiological functions (Valko et al., 2007), but excessive production of ROS and NOS are implicated in various diseases (Carr et al., 2000; Valko et al., 2004; Kovacic et al., 2005; Neo et al., 2010). In our study, ITN should be considered as oxidative agent rather than antioxidant, otherwise high doses exhibiting less antioxidative potentials may be a marker for its oxidative damage by high doses.

Conclusion

ITN exhibited oxidative effect both in in vivo and in vitro studies. The oxidative effect of ITN was found to continue alone and with Vit A which was effectively modulated by Vit C (co-treatment). The modulatory effect of Vit C is due to its powerful antioxidant activity. Both co-treatment groups with Vit C notably reduced the oxidative effect to the tested yeasts, S. Cerevisiae; ITN alone and/or combination with Vit A opposed the effect of Vit C co-treated groups. In addition, ITN in the in vitro antioxidant tests exhibited antioxidant activity in a dose-reduced manners; this confirms ITN to have oxidative effects.

Conflict of interests

The authors have not declare any conflict of interest.

REFERENCES


Isolation and characterization of thermotolerant ethanol-fermenting yeasts from Laos and application of whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis for their quick identification

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Thermotolerant yeasts, which are expected to be applicable for high-temperature fermentation as an economical process, were isolated from four provinces in Laos. Of these yeasts, five isolates exhibited stronger fermentation abilities in a 16% sugars-containing medium of glucose, sucrose, sugarcane or molasses at 40°C than that of Kluyveromyces marxianus DMKU 3-1042, one of the most thermotolerant and efficient yeasts isolated previously in Thailand. One of the five strains, BUNL-17, exhibited the highest ethanol fermentation performance at 45°C. Yeast identification was achieved by whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis as well as by nucleotide sequencing of the D1/D2 domain of the large subunit rRNA gene, revealing that the isolated strains can be categorized into Pichia kudriavzevii, Cyberlindnera rhodanensis and K. marxianus and that all of the five strains are K. marxianus. The results of this study showed that the former analysis is much faster than the latter and reliable and equivalent to the latter.

Key words: Ethanol fermentation, thermotolerant yeast, Kluyveromyces marxianus.

INTRODUCTION

Global energy demand has been continuously increasing due to an increase in the worldwide human population and economic growth. The demand is mostly supplied from traditional fossil fuels, causing a critical elevation of the greenhouse gas level in the Earth’s atmosphere (Talebnia et al., 2010; Ballesteros et al., 2006). Bioethanol is one alternative for fossil fuels and has been widely utilized as a dominant biofuel. Worldwide production of
bioethanol has been increasing over the past 35 years, and the gross output of biofuels including ethanol in 2022 is forecasted to be more than 126 billion liters (Rees, 2014). Considering the convention on biological diversity that includes access to genetic resources and benefit-sharing, useful microbes isolated from own country are beneficial in usability and industrial applicability for each country. Thermostolerant microbes are expected to be crucial for fermentation industries in tropical countries and even in non-tropical countries in summer, because they can be used for high-temperature fermentation, being stably achieved at temperatures around 40°C, which has several advantages including reduction of cooling cost, prevention of contamination and enhancement in enzyme reaction of hydrolysis (Murata et al., 2015). In such fermentation for ethanol production, it is necessary to acquire an efficient yeast strain with a strong tolerance to high temperatures. Mesophilic strains of *Saccharomyces cerevisiae,* however, have been used for a long time in industrial ethanol production. Although there are numerous reports on the potentials of thermostolerant yeast strains, there has been almost no application of them for industrial ethanol production (Kida et al., 1992; Morimura et al., 1997; Sree et al., 2000). The reason(s) is not clear, but it appears that there is still no suitable yeast available.

Many applications have been attempted to isolate thermostolerant yeasts that are capable of growing and fermenting ethanol at high temperatures. Banat et al. (1992) used an enrichment technique for obtaining thermostolerant, fermentative yeasts at 50°C in a medium containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1% glucose. Limtong et al. (2007) enriched thermostolerant yeasts at 35°C in a medium containing 5 or 8% sugarcane juice, 0.05% (NH₄)₂SO₄ and 4% ethanol (pH 4.5), Yuangsaard et al. (2013) used a yeast extract dextrose (YPD) medium supplemented with 4% ethanol at 40°C and Ueno et al. (2001) isolated thermostolerant yeasts from hot spring drainage, Brooks (2008) isolated thermostolerant *S. cerevisiae* from ripe banana peels in Nigeria and Saini et al. (2015) isolated thermostolerant *Kluyveromyces marxianus* strain from local dairies in India. For obtaining thermostolerant yeasts that are applicable for industrial fermentation, the development of a simple and quick screening procedure including species identification is needed.

In this study, an enrichment procedure was applied at a relatively high temperature for screening of thermostolerant and ethanol-fermenting yeasts from samples collected in Laos. Attempt was also made to apply whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis as a very fast procedure for species identification compared to nucleotide sequencing of rRNA genes. The thermostolerance and ethanol fermentation ability of the isolated strains were compared with those of the *K. marxianus* strain DMKU 3-1042 as a control, which is one of the most thermostolerant and efficient strains isolated in Thailand (Limtong et al., 2007).

**MATERIALS AND METHODS**

**Isolation of thermostolerant yeast strains**

Yeasts were isolated from samples of fruits, vegetables, leaves, and soils in four provinces: Luang Phrabang, Xayabury, Xiengkhuang, and Vientiane of Lao People’s Democratic Republic (PDR). Isolation was carried out at 37°C by an enrichment culture. Samples (5 to 10 g) of fruits pressed in small pieces, leaves cut in small portions and mashed soil were transferred into 100-ml Erlenmeyer flasks containing 10 ml of YPD medium containing 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose (Sigma-Aldrich), and incubated at 37°C for 3 days with occasional shaking. The cultures were then streaked on YPD agar plates and incubated at 37°C for 24 to 48 h. The first examination was carried out to test thermostolerance on agar plates of YPD and yeast extract peptone xylose (YPX), which contained 2% xylose (Wako Chemicals) instead of glucose, at different temperatures for 48 h.

**Screening of ethanol-producing thermostolerant yeasts**

Screening for the ability of ethanol fermentation at high temperatures was conducted at 40 and 45°C in 250-ml Erlenmeyer flasks containing 100 ml of YP medium with 16% glucose (YP + 16% D medium) or sucrose (Sigma-Aldrich) (YP + 16% S medium), 16% sugarcane juice supplemented with 0.05% (NH₄)₂SO₄, 0.5% KH₂PO₄ and 0.15% MgSO₄·7H₂O (pH 4.5) (16% sugarcane medium) and 16% molasses supplemented with 0.05% (NH₄)₂SO₄ (pH 4.5) (16% molasses medium). The pre-culture was prepared in YPD medium at 25°C for 18 to 24 h under a rotationally shaking condition at 160 rpm. The pre-culture was transferred at the rate of 5% to each culture medium, followed by incubation at an appropriate temperature under a rotationally shaking condition at 160 rpm.

**Analysis of fermentation parameters**

Cell growth was determined by measuring optical density at 660 nm on a spectrophotometer (Spectrophotometer 258, Corning, New York, USA) after washing twice with distilled water. Ethanol concentration was analyzed by a gas chromatography (Shimadzu GC-9A, Shimadzu, Kyoto, Japan) using polyethylene glycol (PEG-20 M) packed column (length 2.1 m, OD 5 mm, ID 3.2 mm), nitrogen as a carrier gas (35 ml/min), and a flame ionization detector (injection temperature at 200°C, oven temperature at 180°C, detector temperature at 200°C). Sugars as carbon source in media were analyzed by high-performance liquid chromatography apparatus (Hitachi, Japan) with a GLC-810-S Gel pack column (Hitachi) connected to a refractive index detector Model L-2490 (Hitachi) in the mode of 0.5 ml/min eluent of deionized water at 60°C (Rodrussamee et al., 2011).
Identification of isolated strains was carried out by determination of the nucleotide sequence of the D1/D2 domain in the large-subunit rDNA. The genomic DNA was extracted from yeast cells by the slightly modified method of Green and Sambrook (2012). Cells were grown in 3 ml of YPD medium at 30°C under a moderate agitation condition overnight, harvested by centrifugation (14,000 rpm) for 5 min, and resolved in 0.5 ml of sorbitol buffer (1 M sorbitol and 0.1 mM EDTA at pH 7.5). To the cell suspension was added 1 μl of zymolyase solution (5 Ui/μl, Zymo Research) and the suspension was incubated at 37°C for 30 min. Cells were then collected by centrifugation (14,000 rpm) for 1 min and resolved in 0.5 ml of yeast resuspension buffer (50 mM Tris-Cl and 20 mM EDTA at pH 7.5). To the resuspended sample was added 50 μl of 10% SDS and the sample was incubated at 65°C for 30 min. To the incubated sample was added 0.2 ml of 5 M potassium acetate and the sample was kept on ice for 1 h. Cell debris was then removed by centrifugation (14,000 rpm) at 4°C for 5 min. From the supernatant, nucleic acids were recovered as a pellet by centrifugation (14,000 rpm) at 4°C for 5 min after the addition of an equal volume of isopropanol and storage at room temperature for 5 min. The pellet was suspended in 0.15 ml of TE buffer (pH 8.0) containing 30 μg/ml RNase A and incubated at 37°C for 30 min. DNA was then recovered as a pellet by centrifugation (14,000 rpm) for 5 min after addition of 30 μl of 3 M sodium acetate (pH 7.0) and 0.2 ml of isopropanol and storage at room temperature for 5 min. The pellet was dried and suspended in 30 μl of TE buffer, which was used for polymerase chain reaction (PCR) as genomic DNA. PCR was done with a set of forward primer NL-1 and reverse primer NL-4 (O’Donnell, 1993). The PCR products were subjected to agarose gel electrophoresis, purification with a QiA Quick Purification Kit (Qiagen, Ontario, USA) and cycle-sequencing with an ABI BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, California, USA) with the primers NL-1 and NL-4. The nucleotide sequences of samples were then determined on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, California, USA) and compared with those of type strains in databases using the BLAST homology search (Altschul et al., 1990). The nucleotide sequences were aligned using the multiple alignment program CLUSTAL_X version 2.1 (Thompson et al., 1997), and a phylogenetic tree was constructed from the evolutionary distance data with Kimura’s two parameter correction (Kimura, 1980), using the neighbor joining method (Saitou and Nei, 1987) and the MEGA software version 6.0 (Tamura et al., 2013). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

**Whole-cell MALDI-TOF/MS analysis**

Whole-cell MALDI-TOF/MS analysis was performed as described previously (Tani et al., 2015). In brief, a loopful of well-grown yeast (2 to 3 days old, usually 5 to 10 mg in wet weight) on YPD agar plates was suspended in 300 μl of 75% ethanol. The suspension was centrifuged at 15,000 rpm for 2 min. The supernatant was discarded and 50 μl of 70% formic acid was added and mixed. Next, 50 μl of acetonitrile was added and mixed well again. One microliter of the sample was placed onto a spot of a MALDI steel target plate and dried in air. Then 2 μl of matrix solution (saturated solution of sinapinic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was overlaid onto the sample, and the samples were dried in air.

The samples were analyzed with MALDI-TOF/MS (Ultraflex, Bruker Daltonics). Mass spectra were obtained using a positive linear mode in the range of mass-to-charge ratios (m/z) of 2,000 to 20,000. Protein standard comprised insulin ([M+H]+ = 5734.56), ubiquitin-1 ([M+H]+ = 8565.89), cytochrome c ([M+H]+ = 12361.09 and [M+2H]2+= 6181.05), and myoglobin ([M+H]+ = 16952.55 and [M+2H]2+= 8476.77) (Bruker Daltonics).

The obtained spectrum data were further analyzed to create a dendrogram based on spectra similarity using BioTyper software (Bruker Daltonics) under the standard setting (Tani et al., 2015).

**RESULTS**

**Isolation and selection of thermotolerant yeast strains**

In total, 31 thermotolerant yeast strains that could grow on YPD plates at 40°C were isolated by enrichment culture at 37°C with 97 samples, which were collected in four provinces of Laos. Of these strains, 24, four, two strains and one strain were derived from fruits, vegetables, flowers, and soil, respectively. They were further subjected to growth capability tests on YPD and YPX plates at different temperatures (Table 1). DMKU 3-1042, which is one of most thermotolerant *K. marxianus* strains isolated in Thailand (Limtong et al., 2007), was used as a control. DMKU 3-1042 has been extensively analyzed (Rodrussamee et al., 2011; Lertwattanasakul et al., 2011) and its complete genome has been determined (Lertwattanasakul et al., 2015). As a result, nine strains grew well on YPD plates both at 45 and 48°C. On YPX plates, all strains could grow at 40°C, but only five strains grew well at 48°C.

All of the isolates were then used in the experiments to compare ethanol fermentation abilities. They were cultivated in YP +16% D medium at 40°C under a rotationally shaking condition at 160 rpm (Table 2). BUNL-14, 15, 16, 17, 21, and 23 strains exhibited high levels of ethanol production both at 12 h (4.76 to 6.56% (w/v)) and 24 h (5.39 to 7.24% (w/v)), nearly equivalent to or more than that of DMKU 3-1042. Of these strains, BUNL-14 and 17 produced the highest levels of ethanol at 12 and 24 h, respectively. All of these strains except for BUNL-16 were strongly thermotolerant (Table 1). Therefore, five strains, BUNL-14, 15, 17, 21, and 23, as relatively thermotolerant and highly efficient strains were further used in experiments on ethanol fermentation with various sugars.

**Screening of thermotolerant yeasts for ethanol production at high temperatures**

The five strains were cultivated in three media, YP +16% S medium (Table 3), 16% sugarcane (Table 4) and 16% molasses (Table 5), at 40°C under a shaking condition. In the YP +16% S medium, all of the strains except for BUNL-14 showed ethanol production higher than or equivalent to that of DMKU 3-1042 at 12 h, and BUNL-17 showed higher level of ethanol production than those of DMKU 3-1042 at 24 h. In the 16% sugarcane medium, all of the strains produced higher levels of ethanol than those of DMKU 3-1042 at 12 h, and BUNL-14, 15, 17,
Table 1. Growth of the isolated strains on YPD and YPX plates at different temperatures and their identification.

<table>
<thead>
<tr>
<th>Strains</th>
<th>YPD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>YPX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>Temperature (°C)</td>
<td>D1/D2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>BUNL-1</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>BUNL-2</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>BUNL-3</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<td>+++</td>
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<td>+++</td>
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</tr>
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<td>BUNL-9</td>
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<td>BUNL-10</td>
<td>+++</td>
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<td>BUNL-14</td>
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<tr>
<td>BUNL-15</td>
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<td>+++</td>
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<tr>
<td>BUNL-16</td>
<td>+++</td>
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</tr>
<tr>
<td>BUNL-17</td>
<td>+++</td>
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<tr>
<td>BUNL-18</td>
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<td>-</td>
</tr>
<tr>
<td>BUNL-30</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>BUNL-31</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup>+++ strong growth; ++, medium growth; +, less growth; -, no growth. <sup>b</sup>Identification was performed by nucleotide sequencing of D1/D2 domain. NI: Not identified. <sup>c</sup>Identification was performed by the whole-cell MALDI-TOF/MS analysis. Numbers in parentheses are GenBank accession numbers.
Table 2. Ethanol production of the isolated strains in YP + 16% D medium at 40°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentration (% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>BUNL-1</td>
<td>3.23 ± 0.09a</td>
</tr>
<tr>
<td>BUNL-2</td>
<td>2.64 ± 0.13</td>
</tr>
<tr>
<td>BUNL-3</td>
<td>3.04 ± 0.18</td>
</tr>
<tr>
<td>BUNL-4</td>
<td>3.17 ± 0.21</td>
</tr>
<tr>
<td>BUNL-5</td>
<td>3.27 ± 0.03</td>
</tr>
<tr>
<td>BUNL-6</td>
<td>2.84 ± 0.10</td>
</tr>
<tr>
<td>BUNL-7</td>
<td>2.85 ± 0.01</td>
</tr>
<tr>
<td>BUNL-8</td>
<td>3.29 ± 0.48</td>
</tr>
<tr>
<td>BUNL-9</td>
<td>2.84 ± 0.09</td>
</tr>
<tr>
<td>BUNL-10</td>
<td>3.42 ± 0.14</td>
</tr>
<tr>
<td>BUNL-11</td>
<td>1.81 ± 0.03</td>
</tr>
<tr>
<td>BUNL-12</td>
<td>3.59 ± 0.14</td>
</tr>
<tr>
<td>BUNL-13</td>
<td>6.56 ± 0.50**</td>
</tr>
<tr>
<td>BUNL-14</td>
<td>4.94 ± 0.26*</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>4.76 ± 0.13*</td>
</tr>
<tr>
<td>BUNL-16</td>
<td>3.51 ± 0.03</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>3.92 ± 0.54</td>
</tr>
<tr>
<td>BUNL-18</td>
<td>3.72 ± 0.05</td>
</tr>
<tr>
<td>BUNL-19</td>
<td>2.21 ± 0.58</td>
</tr>
<tr>
<td>BUNL-20</td>
<td>5.03 ± 0.61a</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>4.88 ± 0.26*</td>
</tr>
<tr>
<td>BUNL-22</td>
<td>3.72 ± 0.05</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>4.70 ± 0.10*</td>
</tr>
<tr>
<td>BUNL-24</td>
<td>4.82 ± 0.01*</td>
</tr>
<tr>
<td>BUNL-25</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>BUNL-26</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>BUNL-27</td>
<td>0.54 ± 0.00</td>
</tr>
<tr>
<td>BUNL-28</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>5.10 ± 0.50</td>
</tr>
</tbody>
</table>

a ± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; P>0.05). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; P<0.05).

Table 3. Ethanol production of the selected strains in YP + 16% S medium at 40°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentration (% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>BUNL-14</td>
<td>5.03 ± 0.61a</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>4.88 ± 0.26*</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>4.70 ± 0.10*</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>4.82 ± 0.01*</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>5.27 ± 0.01**</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>4.71 ± 0.51</td>
</tr>
</tbody>
</table>

a ± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; P>0.05). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; P<0.05).
Table 4. Ethanol production of the selected strains in 16% sugarcane medium at 40°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentration (% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>BUNL-14</td>
<td>2.89 ± 0.39***</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>2.61 ± 0.02**</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>2.26 ± 0.08**</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>2.68 ± 0.06**</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>2.65 ± 0.08**</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>2.03 ± 0.02</td>
</tr>
</tbody>
</table>

*a± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; *P* >0.05). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; *P* <0.05).

Table 5. Ethanol production of the selected strains in 16% molasses medium at 40°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentration (% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>BUNL-14</td>
<td>0.83 ± 0.46***</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>0.92 ± 0.02**</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>0.85 ± 0.04**</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>0.99 ± 0.18**</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>1.15 ± 0.33**</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

*a± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; *P* >0.05). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; *P* <0.05).

Table 6. Ethanol production of the selected strains in YP + 16% D medium at 45°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentration (% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>BUNL-14</td>
<td>4.14 ± 0.47**</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>4.18 ± 0.25**</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>4.00 ± 0.09**</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>4.35 ± 0.11**</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>4.21 ± 0.25**</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>3.55 ± 0.26</td>
</tr>
</tbody>
</table>

*a± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; *P* >0.05). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; *P* <0.05).

and 21 produced ethanol equivalent to that of DMKU 3-1042 even at 48 h. In the 16% molasses medium, all of the strains produced higher levels of ethanol than those of DMKU 3-1042 at 12 and 24 h, and BUNL-17, 21, and 23 produced ethanol equivalent to that of DMKU 3-1042 at 48 h.

Further fermentation experiments were performed at 45°C with cells being grown in YP + 16% D medium under a shaking condition as shown in Table 6. All of the strains except for BUNL-14 produced higher levels of ethanol than that of DMKU 3-1042 at 12 h. At 24 and 48 h, BUNL-17 showed the highest values of ethanol production though statistically most strains produced ethanol at the levels equivalent to those of DMKU 3-1042. These data suggest that BUNL-17 is the most efficient strain among the isolated strains in YP + 16% D medium at high temperatures.

The five selected strains were identified as *K. marxianus* (see below). They were found to be more efficient ethanol producers than DMKU 3-1042 in glucose,
Table 7. Summary of ethanol production of the selected strains under various conditions.

<table>
<thead>
<tr>
<th>Strains/ Conditions</th>
<th>Time of fermentation</th>
<th>Sugar consumption (% (w/v))</th>
<th>Ethanol (% (w/v))</th>
<th>Ethanol yield (g/g)</th>
<th>Productivity (g/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YP + 16% S medium at 40°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUNL-14</td>
<td>24</td>
<td>16</td>
<td>5.12 ± 0.05</td>
<td>0.32 ± 0.00</td>
<td>2.13 ± 0.02</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>24</td>
<td>16</td>
<td>5.17 ± 0.02</td>
<td>0.32 ± 0.00</td>
<td>2.15 ± 0.01</td>
</tr>
<tr>
<td>BUNL-17</td>
<td>24</td>
<td>16</td>
<td>6.71 ± 0.44**</td>
<td>0.42 ± 0.03**</td>
<td>2.80 ± 0.13**</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>24</td>
<td>16</td>
<td>5.16 ± 0.01</td>
<td>0.32 ± 0.00</td>
<td>2.14 ± 0.01</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>24</td>
<td>16</td>
<td>5.72 ± 0.11*</td>
<td>0.36 ± 0.01*</td>
<td>2.38 ± 0.05*</td>
</tr>
<tr>
<td>DMKU 3-1042</td>
<td>24</td>
<td>16</td>
<td>5.65 ± 0.06</td>
<td>0.35 ± 0.01</td>
<td>2.35 ± 0.25</td>
</tr>
<tr>
<td>16% sugarcane medium at 40°C</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUNL-14</td>
<td>48</td>
<td>16</td>
<td>6.52 ± 0.19*</td>
<td>0.41 ± 0.01*</td>
<td>1.36 ± 0.03*</td>
</tr>
<tr>
<td>BUNL-15</td>
<td>48</td>
<td>16</td>
<td>6.99 ± 0.48*</td>
<td>0.44 ± 0.02*</td>
<td>1.46 ± 0.07*</td>
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<tr>
<td>BUNL-17</td>
<td>48</td>
<td>16</td>
<td>7.31 ± 0.68*</td>
<td>0.46 ± 0.03*</td>
<td>1.52 ± 0.10*</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>48</td>
<td>16</td>
<td>7.31 ± 1.05*</td>
<td>0.46 ± 0.05*</td>
<td>1.52 ± 0.16*</td>
</tr>
<tr>
<td>BUNL-23</td>
<td>48</td>
<td>16</td>
<td>6.46 ± 0.03</td>
<td>0.41 ± 0.00*</td>
<td>1.35 ± 0.01</td>
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<tr>
<td>DMKU 3-1042</td>
<td>48</td>
<td>16</td>
<td>6.64 ± 0.04</td>
<td>0.42 ± 0.01</td>
<td>1.38 ± 0.01</td>
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<td>16% molasses medium at 40°C</td>
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<tr>
<td>BUNL-14</td>
<td>48</td>
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<td>3.48 ± 0.48</td>
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<td>0.73 ± 0.10</td>
</tr>
<tr>
<td>BUNL-15</td>
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<td>8.4</td>
<td>3.5 ± 0.03</td>
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<td>0.73 ± 0.01</td>
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<td>0.45 ± 0.00*</td>
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<tr>
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<td>8.9</td>
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<td>1.94 ± 0.01*</td>
</tr>
<tr>
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<td>24</td>
<td>16</td>
<td>5.39 ± 0.06*</td>
<td>0.34 ± 0.01*</td>
<td>2.25 ± 0.03*</td>
</tr>
<tr>
<td>BUNL-21</td>
<td>24</td>
<td>16</td>
<td>4.80 ± 0.08*</td>
<td>0.30 ± 0.01*</td>
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<td>16</td>
<td>4.71 ± 0.04*</td>
<td>0.29 ± 0.00*</td>
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<tr>
<td>DMKU 3-1042</td>
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<td>16</td>
<td>5.20 ± 0.33</td>
<td>0.33 ± 0.02</td>
<td>2.17 ± 0.14</td>
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</table>

*Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; \( P > 0.05 \)). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; \( P < 0.05 \)).

Sucrose, sugarcane, and molasses media at least in the early incubation period (12 and/or 24 h) at 40°C. They showed different capabilities of ethanol fermentation in different carbon sources: 5.12 to 6.71% at 24 h in YP + 16% S medium (Table 3), 6.46 to 7.31% (w/v) at 48 h in 16% sugarcane medium (Table 4) and 3.45 to 4.02% (w/v) at 48 h in 16% molasses medium (Table 5). BUNL-17 exhibited the highest values of ethanol production among the five strains in YP + 16% D medium at 45°C after 24 h (5.39% (w/v) at 24 h, 4.80% (w/v) at 48 h) (Table 6), and its values of ethanol productivity were greater than those of DMKU 3-1042 in YP + 16% S medium at 24 h and in 16% sugarcane medium at 48 h and in YP + 16% D medium at 45°C at 48 h (Table 7). Notably, BUNL-27, *Cyberlindnera rhodanensis*, showed the highest value of ethanol production (7.79% (w/v) at 24 h) in YP + 16% D medium at 40°C (Table 2), and DMKU 3-1042 showed the highest value of ethanol productivity in 16% molasses medium at 48 h (Table 7). These findings allow us to speculate that there are efficient strains specific for each biomass.

### Identification of some of the isolated strains by nucleotide sequencing

Analysis of the D1/D2 domain of the large subunit rRNA gene was performed for 16 strains, and they were identified as *Pichia kudriavzevii*, *C. rhodanensis* and *K. marxianus* as shown in Table S1 and Table 1. Consistently, phylogenetic tree based on sequences of the D1/D2 domain in the large-subunit rDNA gene demonstrated that eight strains of BUNL-1, BUNL-2, BUNL-4, BUNL-6, BUNL-8, BUNL-10, BUNL-11, and BUNL-25 were located in the same position as *P. kudriavzevii* (Figure 1). Strain BUNL-12 was located in...
Figure 1. Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of the isolated strains with respect to type strain of each species. The phylogenetic tree was constructed as described in Materials and Methods. Numbers indicate percentages of bootstrap sampling, derived from 1,000 samples. The numbers in parentheses are GenBank accession numbers. *Schizosaccharomyces pombe* NRRL Y-12796\textsuperscript{T} was outgroup in the analysis. Bar represents 0.05 K\textsubscript{nuc} distance.

Characterization of isolated strains by whole-cell MALDI-TOF/MS analysis

Whole-cell MALDI-TOF/MS analysis was introduced as a high-throughput identification procedure for identifying known/novel species of bacteria without 16S rRNA gene sequencing (Tani et al., 2015), and it has also been used for characterization of bacteria in ballast water (Emami et al., 2012). This procedure was thus applied for identification of yeast strains isolated in this study, and the resultant spectra data were subjected to clustering analysis using BioTyper software (Figure 2). The analysis revealed that there were three clusters with each cluster including *Pichia kudriavzevii*, *C. rhodanensis* and *K. marxianus* strains that were identified by analysis of the D1/D2 domain of the large subunit rRNA gene (Table S1). This clustering allowed us to identify all of the isolated strains as shown in Table 1. All of the identifications were consistent with colony morphologies described earlier. Taken together, it is concluded that three yeast species were isolated from various samples in Laos by the screening method applied in this study.

DISCUSSION

The acquisition of a thermotolerant fermenting microbe suitable for individual biomass is indispensable for high-temperature ethanol fermentation or for fermentation under temperature-uncontrolled conditions (Murata et al., 2015). A simple and fast procedure for screening of such microbes is thus desired. In this study, two procedures were tested: (1) an enrichment procedure at relatively high temperatures and (2) whole-cell MALDI-TOF/MS analysis for quick identification of species. This, as far as known, is the first large-scale screening of thermotolerant yeasts in Laos as one of the tropical countries.
In respect to fermentation capabilities of isolates reported previously as thermotolerant yeasts, it has been shown that a *Saccharomyces diastaticus* strain produced 6.4% (w/v) ethanol from 15% glucose at 40°C (D’Amore et al., 1989) and a *S. cerevisiae* R-8 produced 4.8% (w/v) ethanol from 10% glucose at 37°C (Brooks, 2008). In *K. marxianus*, a strain DMKU 3-1042 performed the production of ethanol over 6% (w/v) from 22% sugarcane juice at 40°C (Limtong et al., 2007) and a strain TISTR 5925 produced about 4.5% (w/v) from 10% glucose at 42°C (Apiwatanapiwat et al., 2013). Fermentation applications revealed that a strain L.G. produced 3.8% (w/v) ethanol from hydrolyzed 10% Solka-floc (potential glucose content in Solka-floc is 94.5% dry weight basis).
at 42°C by simultaneous saccharification and fermentation (SSF) procedure (Ballesteros et al., 1991) and a strain DBTIOC-35 produced 2.9 (w/v) and 6.2% (w/v) from 10 and 20% biomass (acid pretreated wheat straw), respectively, at 42°C by SSF (Saini et al., 2015). Comparison with these previous achievements reveal that strains isolated in this study, especially BUNL-17 and BUNL-27, have potentials in ethanol fermentation, equivalent to or more than those of strains isolated previously. In addition, fermentation ability has been examined preliminarily with rice hydrolysate as reported previously (Murata et al., 2015). BUNL-17 and DMKU 3-1042 produced over 3% (w/v) and 2% (w/v), respectively, at 40°C in rice hydrolysate medium (equivalent to 10% glucose) supplemented with 0.05% (NH₄)₂SO₄, 0.5% KH₂PO₄ and 0.15% MgSO₄·7H₂O under a shaking condition at 120 rpm. 

As described earlier, whole-cell MALDI-TOF/MS analysis allowed us to divide the isolates to three groups. Strain identification by the two types of analyses matched exactly. It appeared that the thermostolerance and positions of each isolate in the dendrogram did not correlate. It is known that the peaks detected in whole-cell MALDI-TOF/MS analysis are mostly from ribosomal proteins. Thus, the m/z pattern of ribosomal proteins may not be a direct indicator of thermostolerance. Suggestive evidence that divergent copies of ribosomal operons as paralogues in an archaea, Haloarcula marismortui, may improve fitness at high and low temperatures (Lo pez-Lo pez et al., 2007) allowed us to compare the patterns of MALDI-TOF/MS peaks between thermostolerant and less-thermotolerant Kluyveromyces marxianus species reported previously (Lertwattanasakul et al., 2015). However, no significant difference in the patterns was observed between them (data not shown). On the other hand, only three species were obtained in this study. The small variety of species might be due to the application of the enrichment culture in the screening process, in which the major population of microbes may prevent growth of the minor population of microbes. It may thus be necessary to apply a non-enrichment culture for the acquisition of various species.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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REFERENCES


Table S1. Identification of 17 isolated strains by nucleotide sequencing.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Accession number</th>
<th>Closest species with accession number of type species</th>
<th>Nucleotide identity in D1/D2 domain</th>
<th>Nucleotide different in D1/D2 domain</th>
<th>Result of identification</th>
</tr>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td>Identity (%)</td>
<td></td>
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<td>BUNL-1</td>
<td>LC093941</td>
<td><em>Pichia kudriavzevii</em> (EF550222)</td>
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<td>509/510</td>
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<td>BUNL-13</td>
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<tr>
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<td>LC093950</td>
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Full Length Research Paper

Antifungal effects of sisal leaf juice on *Lasiodiplodia theobromae*, the causal agent of mulberry root rot

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This study was carried out to evaluate the antifungal activities of leaf juices (fresh juice, fermented juice, boiled juice and sterile juice) of nine sisal varieties on *Lasiodiplodia theobromae*, the causal agent of mulberry root rot. Results show that all the leaf juices could inhibit the mycelial growth in different degrees (the inhibitory rates ranged from 63.3 to 100%), due to different varieties and treatments. Among the nine varieties, the inhibition effects of hybrid 76416 and *Agave americana* were the best with absolute inhibition of all the leaf juice treatments against the mycelial growth, followed by *Agave Amaniensis*, *Agave virdis*, *Agave angustifolia* and Hybrid 11648. The inhibitory effect of some fresh juices would be cut down after being fermented, boiled and sterilized. The treated mycelia of *L. theobromae* were malformed, enlarged, broken and plasma leaked when observed under the microscope. Most of the leaf juices could inhibit the conidial germination absolutely, except *A. amaniensis*, H.11648 and *A. angustifolia*. The average germination rate of *A. amaniensis*, H.11648 and *A. angustifolia* was 72.4, 16.6 and 13%, respectively. The control efficiency of the fresh juice of H. 11648 against mulberry root rot in the field reached 73.1%.

**Key words:** Sisal, leaf juice, anti-fungi, anti-fungal activities, mulberry root rot, *Lasiodiplodia theobromae*.

INTRODUCTION

Sisal (*Agave sisalana* Perrine) belonging to the Agavaceae family, is an important hard fiber crop popularly grown in tropical and subtropical regions. Sisal is grown in more than 20 countries and the area is about 330 thousand hectares in the world (Huang, 2008). The hybrid 11648 was introduced in China in 1963 and quickly

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became the major variety in south China (Cai, 2000). The sisal culture is one of the main economic activities and has an important social function due to the unfavorable weather and soil conditions in the hilly grounds of Guangxi province, China. Sisal fiber is the main product from this crop, but it only represent approximately 5% of the leaf fresh weight, the remaining 95% including solid and liquid residues (juice of the sisal leaf, containing 81%), are normally discarded by sisal farmers (Oashi, 1999; Suinaga et al., 2006). A number of waste liquid flowed freely around those cutting spots and strong tart flavor and foul smell arose due to the fermentation of the waste which seriously polluted the surroundings. Sisal waste principally contains plant tissue (lignin and cellulose), primary and secondary metabolites such as alkaloids, phenolic compounds, glycosidic saponins, flavonoids, and tannins and water, amongst others (Santos et al., 2009; Chen et al., 2011). In an attempt to utilize this waste, many researches were conducted and researchers have published that the sisal waste or residue contains compounds which can inhibit the growth of Fusarium mangiferae, the pathogen of mango malformation disease (Zhang et al., 2010) and the tumor cells of liver and gastric cancer which caused human death (Hu et al., 2010), poison the larvae of mosquitoes (Pizarro et al., 1999), the gastrointestinal nematodes of goats and sheep (Botura et al., 2011; Roberta et al., 2012), the banana burrowing nematode (Jesus et al., 2015) and the Pomacea canaliculata, a kind of destructive agricultural pest (Li et al., 2012). And also, the sisal waste could be used as fertilizer to improve crop production (Lacerda et al., 2006).

The fungus, Lasiodiplodia theobromae (Pat.) Griffon & Maubl. (Pat.) is pleomorphic, plurivorous and ubiquitous soil-borne pathogen in the tropics and subtropics and is associated with up to 500 plant hosts (Úrbez-Torres et al., 2008). Mulberry root rot caused by L. theobromae in Heng County of Guangxi Province, China, was first reported in 2014 as a new disease and had seriously influenced the healthy and sustainable development of the local sericulture industry (Xie et al., 2014).

Therefore, with a view to controlling the mulberry root rot and making good use of the waste liquid of sisal industry, the antimicrobial activity of sisal leaf juice of nine varieties on L. theobromae, the causal agent of the mulberry root rot was evaluated and the control efficiency of the fresh leaf juice of H. 11648 against mulberry root rot was tested in field in this work.

MATERIALS AND METHODS

Preparation of sisal leaf juice

The fresh juice of nine sisal varieties (Agave amaniensis, Agave fourcroydes, hybrid 76416, Hybrid 11648, Agave angustifolia, Agave virdis, Agave sisalana, Agave americana and hybrid NY1) were extracted from leaves collected from the Germplasm Resources Nursery of Sisal in Nanning, China, by using a juicer. The fermented juice, boiled juice and sterile juice were made by laying fresh juice at 55°C for 20 days, boiling fresh juice for 90 min and sterilizing for 20 min at 121°C, respectively.

Effects of leaf juice on the mycelial growth of L. theobromae

The fresh juice and fermented juice were filtered by bacterial filter (Φ=0.22 μm). Two milliliters of the four treated juices was mixed into 18 ml sterilized and cool PDA medium and poured into Petri dish (90 mm) to make plate, respectively. The 6-mm-diameter mycelia disc from the edge of the actively growing colonies was put on the center of the dishes, then cultured at 28±1°C for 3 days. Each of the treatment was replicated for three times while the control plates (without sisal juice) used equal volume of sterilized water to replace the juice. The mycelial growth of all the treated plates was recorded by measuring the cross diameter of the colonies at three days after inoculation when the upper surface in the control plate was fully covered with the mycelia of L. theobromae. The inhibition percentage was calculated using the formula of Opara and Wokocha (2008):

\[
\text{Inhibition percentage} = \left( \frac{dC - dT}{dC} \right) \times 100
\]

Where, \(dC\) = average mycelial growth of control, \(dT\) = average mycelial growth of treated plates.

Effects of leaf juice on the conidial germination of L. theobromae

For the spore germination test, spores suspension was prepared from pycnidia collected from 20 days old culture of L. theobromae grown on PDA medium with sisal juice of the nine varieties. Sterile distilled water was used to replace sisal juice to make spores suspension for the control. The concentration of all spores suspension treatments were approximately 1.3×10^5/ml and 10 μl suspension was placed on the slide. All the slides were then placed in an inverted position in moist chambers. Each of the treatment was replicated in three times. Spore germination was recorded under microscope in three microscope fields after 15 h of culture and five hundred spores were observed in each field. Percent spore germination was calculated by using the following formula (Sandipan et al., 2014):

\[
\text{Percent spore germination} = \frac{\text{Germination spores}}{\text{Total number of spores}} \times 100
\]

Field experiment of leaf juice against mulberry root rot

According to the above inhibition results, fresh leaf juice (leaf liquid residue) of a H. 11648 was used to test the field control efficiency against mulberry root rot. The field experiment was conducted from April 2014 to August 2015 in Heng county, Guangxi province, China. The diseased plants and dead plants in the experimental mulberry yard were surveyed and confirmed to be the root rot trees by surveying the symptoms and identifying the pathogens before sprinkling 25 kg leaf juice on the superficial soil near mulberry plants of each plot at the first time. The diseased and dead rate of mulberry root rot in the experimental yard was about 10%. Each row (about 10 m long, containing 80 plants) of the yard was set to be an experimental plot and five plots of every treatment were prepared that is, five replicates. The juice was used once a month and the control plots were sprinkled with water in equal volume. The plants were calculated again at 15 days after the last time of sprinkling leaf juice and the diseased or dead trees were clarified to be caused by the pathogen of the mulberry root rot. The control
Table 1. Inhibition rates of four treated juice of nine sisal varieties against the mycelia growth of *L. theobromae*.

<table>
<thead>
<tr>
<th>Sisal varieties</th>
<th>Juice concentration (ml/10 ml)</th>
<th>Juice treatments and inhibitory effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fresh juice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>A. amaniensis</td>
<td>1.00</td>
<td>0.80±0.01f</td>
</tr>
<tr>
<td>A. fourcroydes</td>
<td>1.00</td>
<td>3.00±0.03b</td>
</tr>
<tr>
<td>Hybrid No.76416</td>
<td>1.00</td>
<td>0.00±0.00g</td>
</tr>
<tr>
<td>H.11648</td>
<td>1.00</td>
<td>2.00±0.01d</td>
</tr>
<tr>
<td>A. angustifolia</td>
<td>1.00</td>
<td>1.30±0.01e</td>
</tr>
<tr>
<td>A. virdis</td>
<td>1.00</td>
<td>0.08±0.01f</td>
</tr>
<tr>
<td>A. sisalana</td>
<td>1.00</td>
<td>2.60±0.06c</td>
</tr>
<tr>
<td>A. americana</td>
<td>1.00</td>
<td>0.00±0.00g</td>
</tr>
<tr>
<td>Hybrid NY1</td>
<td>1.00</td>
<td>2.60±0.00c</td>
</tr>
<tr>
<td>CK</td>
<td></td>
<td>8.80±0.03a</td>
</tr>
</tbody>
</table>

Data showed in the table are the average of three replicates. D, Average mycelia growth diameter of treated plates (cm); R, relative inhibitory rate (%). Different lowercases letters in the same column represent significant difference at 5%.

Effects were evaluated by using the following formulas (Lai, 1998) and made some appropriate improvements:

\[
\text{Diseased and dead percentage (DDP)} = \frac{\text{Diseased plants} + \text{dead plants}}{\text{Total plants}} \times 100
\]

\[
\text{Control effects percentage} = \frac{\text{CDDP of control plots} - \text{CDDP of treated plots}}{\text{CDDP of control plots}} \times 100
\]

\[
\text{Correcting DDP (CDDP)} = \text{DDP after sprinkling juice} - \text{DDP before sprinkling}
\]

All the collected data in this work were statistically analyzed by using DPS (Version 7.05) software and means were separated by Duncan multiple range test at 5% probability level.

**RESULTS**

Inhibition of sisal leaf juice on the mycelial growth of *L. theobromae*

All the sisal juice of nine varieties could inhibit the mycelial growth of *L. theobromae* significantly with the inhibitory rates ranging from 63.4 to 100%, especially the juice of hybrid No. 76416 and *A. americana* which inhibited the mycelial growth absolutely (Table 1). The inhibition effects of *H. 11648* and *A. angustifolia* were at a middle level and the inhibitory rates of four juice treatments were 91.5, 85.4%, 82.9, 80.5% and 85.4, 79.3, 79.3, 76.8%, respectively. The inhibitory rates of *A. sisalana*, *A. fourcroydes* and hybrid NY1 ranged from 63.4 to 75.6%; had lower inhibition effects than others. In fresh juice treatments, the inhibition rates of hybrid No. 76416 and *A. americana* were the highest (100%) followed by *A. amaniensis* (97.6%) and *A. virdis* (97.6%) and the inhibition rate of *H. 11648* was 85.4%. Though the juice of *A. fourcroydes* was the worst among the nine varieties, its inhibition rate (73%) was still significantly higher than the control. Inhibition effects of fresh juice of some varieties (*A. virdis, A. fourcroydes, A. angustifolia, H.11648, A. sisalana* and hybrid NY1) would be cut down a little while being fermented, boiled and sterilized, but the inhibitory rates of the four treatments of hybrid 76416, *A. americana* and *A. amaniensis* would not be changed and kept at 100, 100 and 97.6%, respectively. The colonies of *L. theobromae* were blackened with dense substrate mycelia and little aerial mycelia in most treatments (Figure 1). The mycelium was enlarged and malformed (*A. americana, A. angustifolia, A. sisalana*, etc.), broken (H. *11648, A. fourcroydes, A. virdis*, etc.), broken with protoplasm leaked (*H. 11648, A. americana, A. amaniensis*, etc) (Figure 2).

Inhibition of sisal leaf juice on the conidial germination of *L. theobromae*

Except the juice of *A. amaniensis, H.11648* and *A. angustifolia*, the four treated juice of the other six varieties could inhibit the conidial germination absolutely. Although, the conidia could germinate in the juice of *H.11648* and *A. angustifolia*, the
Figure 1. The colonies of *L. theobromae* on the PDA media treated with different sisal juice.

Figure 2. Mycelia characteristics of *L. theobromae* treated with leaf juice of sisal. **A.** Enlarged and malformed mycelia. **B.** Broken mycelia. **C.** Broken mycelia with protoplasm leakage. **D.** Normal mycelia.
Table 2. Conidia germination rates of *L. theobromae* in different treatments of sisal leaf juices.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Fresh juice</th>
<th>Fermented juice</th>
<th>Boiled juice</th>
<th>Sterile juice</th>
<th>Average germination rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. amaniensis</em></td>
<td>70.40±0.16b</td>
<td>72.60±0.21b</td>
<td>72.00±0.14b</td>
<td>74.40±0.15b</td>
<td>72.40±0.13b</td>
</tr>
<tr>
<td><em>A. fourcroydes</em></td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Hybrid No.76416</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>H.11648</td>
<td>14.00±0.06c</td>
<td>15.40±0.17c</td>
<td>17.60±0.04c</td>
<td>19.20±0.12c</td>
<td>16.60±0.15c</td>
</tr>
<tr>
<td><em>A. angustifolia</em></td>
<td>10.80±0.09d</td>
<td>12.60±0.11d</td>
<td>12.00±0.10d</td>
<td>16.60±0.21d</td>
<td>13.00±0.08d</td>
</tr>
<tr>
<td><em>A. virdis</em></td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td><em>A. sisalana</em></td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td><em>A. americana</em></td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Hybrid NY1</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>CK</td>
<td>98.40±0.07a</td>
<td>98.40±0.07a</td>
<td>98.40±0.07a</td>
<td>98.40±0.07a</td>
<td>98.40±0.07a</td>
</tr>
</tbody>
</table>

Data shown in the table are the average of three replicates. Different lowercase letters in the same column represent significant difference at 5%.

Table 3. Control efficiency of fresh juice of H. 11648 against mulberry root rot in field.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>DDP before sprinkling juice</th>
<th>DDP after sprinkling juice</th>
<th>CDDP</th>
<th>Control efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf juice</td>
<td>14.54±0.05%b</td>
<td>18.78±0.05%b</td>
<td>4.28±0.03%b</td>
<td>73.10%</td>
</tr>
<tr>
<td>CK</td>
<td>15.56±0.06%a</td>
<td>31.47±0.02%a</td>
<td>15.91±0.03%a</td>
<td></td>
</tr>
</tbody>
</table>

Different lowercase letters in the same column represent significant difference at 5%. DDP, diseased and dead percentage, CDDP, correcting DDP.

germination rates were very low. The average conidial germination rate in the fresh juice of H. 11648 and *A. angustifolia* was 16.6 and 13% respectively. But the conidia in the juice of *A. amaniensis* had relatively high germination rates (above 70%) and the average germination rate of four treated juice was 72.4% (Table 2).

**Control efficiency of fresh leaf juice of H. 11648**

As mentioned before, H. 11648 is the main cultivated variety and others are just planted and preserved as germplasm resources in China and, the leaf juice of H. 11648 could significantly inhibit the mycelial growth and conidial germination of *L. theobromae*. Therefore, the fresh leaf juice of H. 11648 collected from the Shanxu Farm of Guangxi State Farms was used for the field experiment due to its adequate sources and significant inhibitions. Results show that the fresh leaf juice of H. 11648 had significant control efficiency (73.1%) against the disease after continuously sprinkling of the juice for about 16 months (Table 3).

**DISCUSSION**

The search for natural products from plants and agro-industrial waste, which may become useful to society, has been the subject of intense research in recent years (Harvey, 2007; Lee et al., 2007), and botanical pesticides with low toxicity, little or no residue and environment-friendly due to the source of the plant itself have become the highlight of researchers (Corato et al., 2010; Wang et al., 2012). The anti-fungi activities of ethanol extracts of 77 plants containing *Alpinia chinensis*, *Eleutherrine plicata*, *Vatica xishuangbannensis*, *Morindu cochinchinensis* and *A. americana* against 12 plant pathogenic fungi were studied; results showed that the ethanol extracts of 15 plants had inhibitory effects on the target pathogens and the extracts of *A. americana* could significantly inhibit the mycelia growth of *Exserohilum turcicum* with the inhibition rate of 76.6% (Zhang et al., 2011). The extracts of *Acalypha hispida*, *Chromolaena odorata*, *Azadirachta indica* and *tetraptera tetrapleura* could effectively inhibit the mycelia growth of *L. theobromae* and the inhibition rates ranged from 30.2 to 88.44% (Adeniyi and Joseph, 2015). Ethanol extracts and anthraquinones isolated from the root extract of *Coccoloba mollis* showed anti-fungi activity against *L. theobromae*, the inhibition rate of the most active compound (emodin) was up to 44% (Barros et al., 2011). As a cosmopolitan soil-borne fungus, *L. theobromae* could cause both field and storage diseases on more than 280 plant species including fruits, crops and
plantation trees (Talukdar, 1974; Singh et al., 1977; Domsch et al., 1980). In most cases, the pathogen and its induced diseases were managed by chemical fungicides, such as Dithane M-45 (Jayanta and Raj, 1989; Bhadra et al., 2014), carbendazim and thiophanate methyl (Banik et al., 1998; Mahmood and Gill, 2002; Shahbaz et al., 2009; Sultana and Ghaffar, 2010) but chemical fungicides may cause phytotoxicity to the mulberry trees and poison the silkworms due to the high sensitive to many chemicals. Therefore, searching for the botanical fungicide and applying biocontrol become very important in controlling the diseases of mulberry. In our work, leaf juice of sisal was used for antifungal tests and showed promising results for their use as fungicides and all leaf juices of the nine sisal varieties had strong antifungal activities against L. theobromae. The results also revealed that anti-fungi compounds extensively existed in the plants of Agave. The fresh juice of H. 11648 could effectively control the mulberry root rot rot in field. Leaf juice of sisal made the mycelia of L. theobromae to be enlarged, malformed, broken and protoplasm leaked, which is similar to the antagonistic mechanisms of some biocontrol microbes (Brain et al., 1945; Chen et al., 2009; Ikeda et al., 2012; Levy et al., 2015). Different inhibition effects on the target pathogen indicated that the concentrations of the antifungi active substances contained in those leaf juices might be different. Antifungal activities of some leaf juices would be cut down after being fermented, boiled and sterilized, but the others would not be changed, indicating that differences existed in the types and physicochemical properties of the antifungi substances in the leaf juices extracted from the same variety and the different varieties; some antifungal substances have thermal stability and others do not. The result of the present work is similar to that of Zhang et al. (2010). So, it may not be a simple antifungal substance but a kind of antifungal composites or compounds contained in the sisal leaf juice. Promising purification work of the antifungal compounds from sisal waste will be conducted in subsequent studies.

**Conclusion**

Liquid residue (leaf juice), the by-product of sisal industry showed significant anti-fungal activities on L. theobromae and control efficiency against the mulberry root rot disease, which revealed a new way not only for raising the reasonable and comprehensive utilization of sisal waste, but also for controlling the mulberry root rot and other diseases caused by L. theobromae.

**Conflict of interests**

The authors have not declared any conflict of interest.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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