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## Full Length Research Paper

## Bioactivity of ethanolic extracts of *Euphorbia pulcherrima* on *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

Viviane Tavares Almeida<sup>1,2\*</sup>, Vânia Maria Ramos,<sup>1,2</sup> Matheus Barbosa Saqueti<sup>1,2</sup>, Pedro Henrique Gorni<sup>1</sup>, Ana Cláudia Pacheco<sup>1</sup> and Renato Marcos de Leão<sup>1,2</sup>

<sup>1</sup>Agronomy Department, College of Agricultural Sciences, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil.

<sup>2</sup>Laboratory of Agricultural Entomology, College of Agricultural Sciences, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil.

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The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is a polyphagous species which attacks many economically important crops in several countries. This insect is an important pest of corn, and currently the most widely used control method is chemical. In order to minimize environmental impacts, other forms of control have been tried, and accordingly, the investigation of plants with insecticidal effects becomes relevant. Thus the present study was conducted in order to evaluate the effect of the ethanolic extract of *Euphorbia pulcherrima* (poinsettia) leaves in fall armyworm biology. Extracts were prepared from leaves of the plant *E. pulcherrima* collected at different phenological stages (vegetative and reproductive), oven dried, crushed and then solubilized in ethanol, yielding the ethanol extract. The extracts were set aside in 0.5 and 1% concentrations for each phenological stage of the plant, incorporated into an artificial diet and offered to the larvae of *S. frugiperda*. The extract of vegetative and reproductive phase of *E. pulcherrima* leaves in concentrations of 0.5 and 1%, has showed that it affected mortality in the larvae, increasing the larval period and reducing the weight of larvae and pupae and viability of the eggs of the caterpillars. Ethanolic extract of *E. pulcherrima* leaves in the reproductive phase of the plant is effective to reduce the *S. frugiperda* population.

**Key words:** Botanical insecticide, plant extracts, mortality, poinsettia, pest biology.

### INTRODUCTION

Among all species of insect pests, some stand out because of the negative impact they cause to agribusiness (attack crops with higher planted area) and

the differentiated amount of crops that attack (Zarbin and Rodrigues, 2009). According to these criteria, seven of the top ten pest species are of the Lepidoptera order

\*Corresponding author. Email: [vivianetavaresdealmeida@gmail.com](mailto:vivianetavaresdealmeida@gmail.com) Tel: (055 18) 99719-0723.

(moths): *Spodoptera frugiperda* (J.E. Smith), *Spodoptera eridania* (Cramer), *Mocis latipes* (Guenée), *Agrotis ipsilon* (Hufnagel), *Corcyra cephalonica* (Stainton), *Plodia interpunctella* (Hubner), *Elasmopalpus lignosellus* (Zeller), *Procornitermes triacifer* (Silvestri), *Diabrotica speciosa* (Germar) and *Acromyrmex landolti* Forel (Zarbin and Rodrigues, 2009). Among these pests, the main pest of Brazilian agriculture can be considered the moth *S. frugiperda*, also known as fall armyworm, due to attack different crops, especially grasses (Vendramim et al., 2000), which together represent 97% of all planted area in the country. Their outbreaks have caused significant losses also in crops such as cotton, soybean and cultivated Solanaceae members (Pogue, 2002; Barros et al., 2010), besides using alternative hosts to remain in agricultural ecosystems.

The control methods of this insect focus primarily on the use of synthetic insecticides of high cost and with high risk of toxicity and environmental contamination (Viana and Prates, 2003). Therefore, it has been carried out researches on control measures with less environmental impact, and in this sense, the plants emerge as an important alternative for the management of this pest. According to Torres et al. (2001), natural products extracted from plants are sources of substances which may be used in pest control, being compatible with integrated pest management programs (IPM) as an option to minimize the negative effects of indiscriminate use of insecticides.

Currently, there are several researches involving insecticide plants in the control of *S. frugiperda*, which show promising results (Viana and Prates, 2003; Santiago et al., 2008). By testing various aqueous extracts of Meliaceae on *S. frugiperda*, Góes et al. (2003), revealed the existence of some plants with toxic activity, highlighting, among them *Trichilia pallida*, in addition to *Azadirachta indica* extract, which prevents the insect molting, leading them to death. Another action mechanism of the active ingredients from botanical insecticides is to affect certain organs or insect molecules, and in this case, it is act hindering the growth and development by interfering with cellular metabolism (Aguar-Menezes, 2005). Depending on the concentration used, some extracts can reduce the viability of eggs, nymphs, larvae and pupae. The reduction of the eggs and the oviposition inhibition are important effects from plant extracts on the reproduction of insects (Costa et al., 2004).

The aim of this study was to evaluate the effect of ethanolic extract of *E. pulcherrima* leaves collected in different phenological stages on *S. frugiperda*.

## MATERIALS AND METHODS

### Trial place

The experiment was conducted at Universidade do Oeste Paulista

(UNOESTE) in Presidente Prudente (22°7'39" S, 51°23'8" W, 471 m.a.s.l.) São Paulo, Brazil, in the laboratory of Agricultural Entomology (LEA), using a room with controlled temperature of 26.0°C ± 1.0°C, humidity 60% ± 10% and 12 h photoperiod. The caterpillars used in the experiment were reared in the laboratory from the company BUG - Agentes Biológicos®.

### Obtaining extract

To obtain the extract, fully expanded leaves of *E. pulcherrima* were collected in the plants hatchery of the Universidade do Oeste Paulista (UNOESTE) in vegetative and reproductive phases. The leaves were stored in kraft paper bags and dried in a kiln at 60°C for 48 h and therefore crushed (grinded) in a knife mill (Willye®) to a particle size of 0.45 mm, to obtain a fine powder that was then stored in sealed glass containers and kept at 24°C in a dark room until the manipulation of the extracts.

The obtained plant powder was macerated in ethanol solution and filtrated once a week. The filtering was performed on a conventional glass funnel, using as filter germination paper. After filtering, the ethanol was replaced in the bottle until it covers 4 cm of the volume filled by the powder. This procedure was performed to exhaustion to obtain the ethanolic extract (Santana et al., 2013).

The obtained solvent was evaporated under reduced pressure on a rotary evaporator (Quimis – Q344B), a procedure for obtaining the pure ethanolic extract. The extracted content was stored according to each phenological stage of the plant and spared to be added to the artificial diet (Parra, 1999).

### Application of extract

The extract was weighed in the amounts of 5 and 10 g, respectively, corresponding to concentrations of 0.5 and 1% (w/v), which were added in 1 L of the diet, forming 5 treatments according to Table 1. The mixture was poured into gerbox containers, which stood for 40 min in the laminar flow hood with UV light for germicidal function, and soon after it was stored in the refrigerator until the inoculation of the larvae.

After preparation and cooling, the diet was cut into cubes containing on average 4 g of diet. The cubes were added individually in plastic pots of 75 ml, after that, larvae in second instar were placed under artificial diet *ad libitum*. Each treatment consisted of 50 repetitions, each repetition using one of the larvae.

### Evaluated parameters

Observations were made every day to record the larval and pupal mortality. The caterpillars were weighed on the 3rd, 6th, 9th and 12th day after the start of the experiment (Precision Scale - Shimadzu AUJ 220). Twenty four hours after the formation of pupae, they were observed under a binocular microscope for determining the sex (Butt and Cantu, 1962), and then they were weighed and placed in Petri dishes. Immediately after the formation of pupae the remaining diet and feces were weighed to determine the food intake and weight of stools.

For each treatment, seven male and female the pupae were used of same age to form seven moths couples of same age and placed inside of PVC cages (10 cm diameter x 15 cm high) coated with filter paper for oviposition purpose. Each pair was fed with an aqueous solution containing 10% honey. The cages were covered at its upper end with "voil" fabric and its base closed with plastic wrap and adhesive tape.

Every day, the egg masses were collected, transferred to plastic pots of 75 ml and stored in a room at 26°C, 60% of relative humidity and 12 h of photoperiod. The eggs of the second mass were

**Table 1.** Treatments containing extract of the leaf of *Euphorbia pulcherrima*, offered in an artificial diet for *Spodoptera frugiperda* larvae.

Treatment	Extract (%)	Phenological stage
TT	0	-
VE0.5	0.5	Vegetative
VE1	1.0	Vegetative
RE0.5	0.5	Reproductive
RE1	1.0	Reproductive

TT (control), VE0.5% and VE1% (extract of the vegetative phase), RE0.5% and RE1% (extract of the reproductive phase).

**Table 2.** Total mortality (%) and larval period (days) of *S. frugiperda* larvae fed with an artificial diet containing ethanolic extract of *E. pulcherrima* leaves.

Extract	Mortality (%)	Larval period (days)
TT	0 <sup>a</sup>	13.816±0.119 <sup>b</sup>
VE0.5%	22±0.059 <sup>b</sup>	15.820±0.328 <sup>b</sup>
VE1%	12±0.046 <sup>b</sup>	19.045±0.425 <sup>a</sup>
RE0.5%	24±0.061 <sup>b</sup>	19.657±0.712 <sup>a</sup>
RE1%	26±0.062 <sup>b</sup>	18.432±0.588 <sup>a</sup>
P-value = 0.001864692		P-value = 1.1791E-21

Means followed by the same letter in the column do not differ significantly by the Kruskal-Wallis test at 1%.

counted, placed in plastic bowls (75 mL) until the eggs hatch, in order to measure their viability.

#### Experimental design and statistical analysis

The experimental design was completely randomized with 5 treatments. In the test with the caterpillars they were 50 repetitions and in the test with moths couples the number of repetition was 7.

After that, all the parameters were submitted to Shapiro-Wilk test; then it was performed nonparametric means comparison by Kruskal-Wallis test, using Action 2.9 program (Estatcamp, 2015).

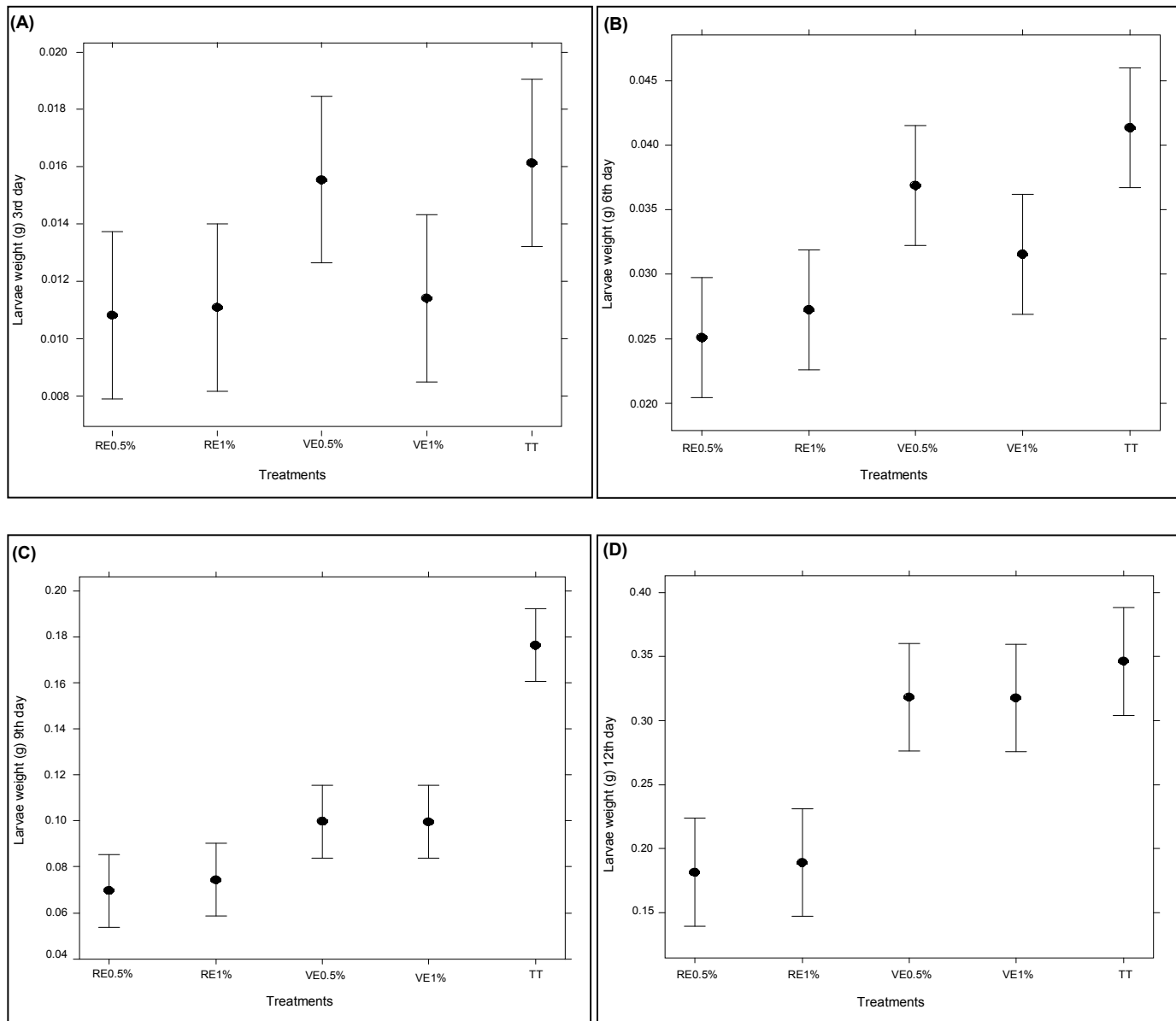
## RESULTS AND DISCUSSION

The ethanolic extract of *E. pulcherrima* leaves collected in the two phenological phases of the plant (vegetative and reproductive) with 0.5 and 1% concentration have caused higher larvae mortality compared to the control (Table 1). D'Incao et al. (2012) have reported that the cold aqueous extract of *E. pulcherrima* applied to leaf discs of *Neonotonia wightii* (perennial soybean) caused 58.5% mortality of *S. frugiperda*. Soares et al. (2011) have observed the influence of *Rosmarinus officinalis* 10% essential oil on *S. frugiperda* found mortality of 30%. The mortality value displayed can be correlated with the concentrations used in this study; perhaps higher

concentrations produce greater larvae mortality rate. Prates et al. (2003) have studied the correlation among different concentrations of neem leaf aqueous extract (*A. indica*) on *S. frugiperda* mortality and concluded that the increasing concentration in this product leads to increased mortality rate.

Regarding to the larval period, the treatments VE1%, RE0.5% and RE1% caused a prolongation on the larval stage of 5.2, 5.8 and 4.7 days, respectively, compared to the other treatments (Table 2). According to Torres et al. (2001) this extension may be related to the presence of growth inhibitors, low conversion of ingested food or by contain toxic substances that interfere with the lower food intake. This result is corroborated by Piubelli (2004) and Hoffmann-Campo et al. (2006) who have studied biological aspects of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), fed with diets with the addition of the rutin flavonoid, observed an extension in feeding time.

The extension of the larval period in the field can leave the insects vulnerable for longer periods to parasitoids, predators and entomopathogenic organisms attack. The emerging adults may be in asynchrony compared to the normal population, and consequently copulation could be more difficult or lead to inbreeding by mating individuals of the same generation (Rodríguez and Vendramin, 1996). The number of insect generations in the



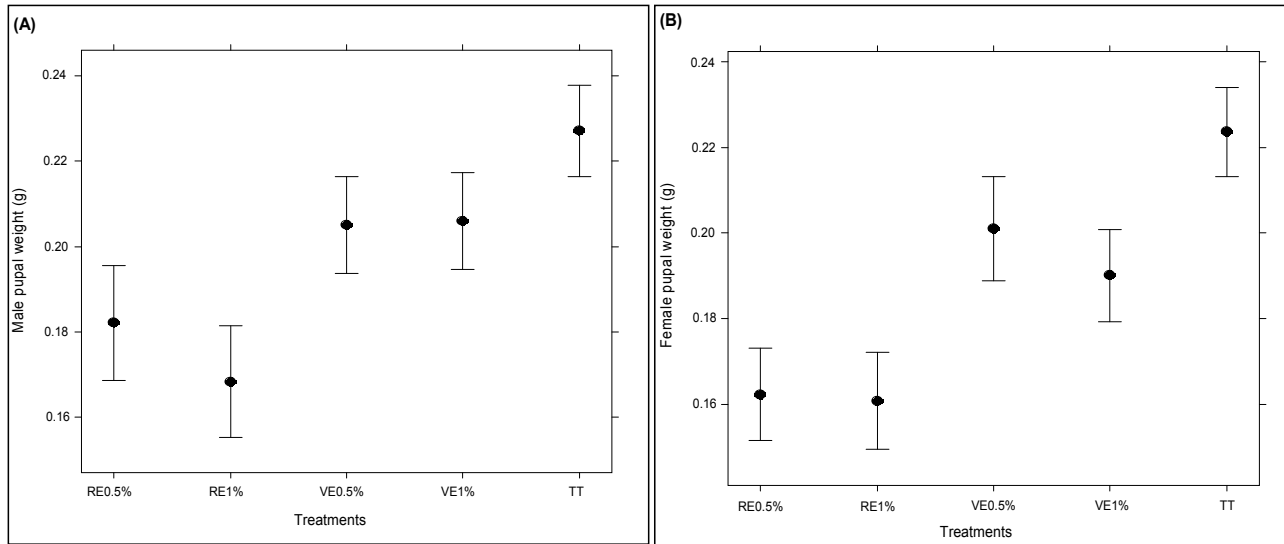
**Figure 1.** Weight (g) of *S. frugiperda* larvae on the 3rd, 6th, 9th and 12th day, feeding with artificial diet containing *E. pulcherrima* extracts from leaves. Legend: TT (control), VE0.5% and VE1% (extract of the vegetative phase), RE0.5% and RE1% (extract of the reproductive phase). P-value <0.01 by Kruskal-Wallis test.

agricultural cycle can be reduced, as was stated by Tanzubil and McCaffery (1990).

In the 3<sup>rd</sup>, 6<sup>th</sup>, 9<sup>th</sup> and 12<sup>th</sup> day the caterpillars were weighted to check the gain and/or weight loss ethanolic extracts. On the 3<sup>rd</sup> day there was no difference among treatments, probably because of the molecules present in treatments have not yet been metabolized by the caterpillars (Figure 1a). From the 6<sup>th</sup> day increased weight was observed for the control treatment (0.041g), showing the influence of the extracts on the larvae weight fed with VE0.5% (0.036g), VE1% (0.031g), RE0.5% (0.025g) and RE1% (0.027g) (Figure 1b). It can be affirm

that the RE0.5% treatment was statistically superior to treatment VE0.5%, since there was 30% reduction in weight.

The body weight of the 9<sup>th</sup> day, it was again confirmed the influence of the extracts, verifying that the control treatment showed the highest body weight (0.176 g) and treatments VE-0.5%, VE-1%, RE-0.5%, and RE-1% led to reduced weights for 0.099, 0.099, 0.074 and 0.069 g, respectively (Figure 1c). Similarly, Bogorni and Vendramim (2003) using extracts from six species of *Trichilia* spp. comparing with *A. indica* (neem), have also found reduced weight for *S. frugiperda* in treatments



**Figure 2.** Weight (g) of male and female pupae of *S. frugiperda*, feeding at the larval stage with artificial diet containing *E. pulcherrima* extracts from leaves. Legend: TT (control), VE0.5% and VE1% (extract of the vegetative phase), RE0.5% and RE1% (extract of the reproductive phase). P-value <0.01 by Kruskal-Wallis test.

where neem seed extracts and *T. pallens* leaves were used.

Growth inhibition and poor weight gain can be attributed to reduced feeding and impaired ability to convert nutrients into biomolecules to form the tissues of insects, preventing growth and weight gain (Martinez and Emden, 2001). For Tanzubil and McCaffery (1990), significant weight reduction caused by the extracts indicates that the insects, in need of degrading possible secondary metabolites present in the extracts, can be diverted for this purpose, resources that would be used to gain biomass.

On 12<sup>th</sup> day, the last weighting was made, and the treatments RE0,5% and RE1% resulted in larvae with lower weights in relation to other treatments, being 0.181 and 0.189 g the results, respectively. Control, VE-0.5% and VE-1% the respective weight was 0.346, 0.318 and 0.317 g (Figure 1d). As in the 6<sup>th</sup> day, it was observed that the treatment of reproductive stage containing *E. pulcherrima* extract with the concentration of 0.5% was superior to the treatment with the highest concentration (1%) of the leaf extract collected in the vegetative phase. This can be attributed to the amount of metabolites present in the leaves, since different phenological stages of plants influence the amount and the dynamic of secondary metabolites that are present in them (Gobbo-Neto and Lopes, 2007). Tavares et al. (2005) analyzed the essential oil of three chemo types of *Lippia alba* and found an increase in the percentage of limonene during the flowering season, however the percentage of citral, carvone and linalool has slightly decreased during the reproductive phase.

Németh et al. (1993) have studied wild specimens of

*Achillea crithmifolia* under different environmental conditions and developmental stages; they found that the rate of camphor in the essential oil decreased as the plants have advanced in their phenological stages. For the essential oil 1.8-cineol, the observed behavior was the opposite.

Based on the current study we can conclude, that, it is more efficient to use the extract made from the leaves of plants in the reproductive stage, since the caterpillars had reduced body weight.

Regarding the weight of pupae, there was a reduction in male pupae weight for treatments RE-0.5% (0.182 g) and RE-1% (0.168 g) compared to treatments with VE-0.5% and VE-1% which showed weight of 0.205 g, and the control treatment that obtained the highest weight (0.227 g) (Figure 2a). In the same direction, the weight of females pupae were significantly higher in the control treatment (0.223 g), followed by treatments VE-0.5% (0.201 g) and VE-1% (0.190 g). The treatments RE-0.5% (0.182 g) and RE-1% (0.168 g) have caused the greatest reduction of the weight of female pupae (Figure 2b). Ramos-López et al. (2010) observed a gradual decrease in weight of *S. frugiperda* pupae exposed to different extracts of *Ricinus communis* (Euphorbiaceae). Several studies using plant extracts on *S. frugiperda* reported reduction in pupae weight (Rodriguez and Vendramim, 1996; Vendramim and Scampini, 1997; Roel et al., 2000). However, D'Incao et al. (2012), evaluating the average weight of *S. frugiperda* pupae observed that the cold aqueous extract of *E. pulcherrima* applied to leaf discs of *Neonotonia wightii* (perennial soybean) was not different compared to the control.

The weight reduction in the pupal period is probably

**Table 3.** Food consumption (g) and weight of stool (g) of *S. frugiperda* larvae, fed with artificial diet containing ethanolic extract of *E. pulcherrima* leaves.

Extract	Food consumption (g)	Weight of feces (g)
TT	3.125±0.152 <sup>a</sup>	1.023±0.068 <sup>a</sup>
VE0.5%	3.073±0.084 <sup>a</sup>	0.856±0.051 <sup>a</sup>
VE1%	3.113±0.114 <sup>a</sup>	1.018±0.053 <sup>a</sup>
RE0.5%	3.034±0.087 <sup>a</sup>	0.623±0.081 <sup>b</sup>
RE1%	3.213±0.079 <sup>a</sup>	0.630±0.049 <sup>b</sup>
	P-value = 0.314352123	P-value = 4.51015E-07

Means followed by the same letter in the column do not differ significantly by the Kruskal-Wallis test at 1%.

related to the effects of substances in plant extracts ingested by the caterpillars during the larval stage. The toxic effect of insecticidal plants affects usually more larval stages than pupal stages, due to the fact that caterpillars are going to ingest the nutrients present in the food (Rodriguez and Vendramim, 1996; Céspedes et al., 2000; Martinez, 2001). This effect reflects in the insect morphology, reducing the weight of male and female pupae, as found in this study. The feeding reduction or low food conversion caused by plant extracts may interfere in the pupae weight. If the weight is lower than the control, it is suggested that the chemical compounds present in the plant might have caused a decrease in food consumption by the larvae. Consequently, pupae with low weight would become small adults, and possibly there will be problems in the mating behavior of these individuals compared to those with normal, weight pupae, leading to less females fertilized (Rodriguez and Vendramim, 1996).

After weighting the pupae, the food and feces that were left over in the pots were also weighted to measure food consumption and stool weight. Food intake was not affected by the treatments, but the caterpillars that received treatments containing extract of *E. pulcherrima* leaves in reproductive stage were reduced by 40% (RE-0.5%) and 39% (RE-1%) of excreted feces comparing to the control (Table 3). Probably, this result has occurred because the extracts have affected the digestibility of food by *S. frugiperda*. So, despite the fact that the larvae have fed normally, the food was kept for longer time in the gut for degradation of secondary metabolites present in the extracts. Similar type of results were presented by Sâmia (2013) using *Copaifera langsdorffii* aqueous extracts in 2nd instar *S. frugiperda* larvae, leading to reduced weight of feces excreted; the author states that this reduction may be related to food deterrence caused by some substance present in aqueous extracts, possibly enzyme inhibitors. Tirelli et al. (2010) using tannic fractions of *Schinus terebinthifolius* have found a reduction in excreted feces in the control treatment, but these treatments did not decrease the food consumption.

It is interesting to note that, despite the regular amount of food intake in larval stages, because the applied extracts of *E. pulcherrima* did not have proper utilization of the food, since its weight has been reduced.

Observing the fecundity of *S. frugiperda*, even not perceived any statistical significance, the number of eggs and egg masses decreased in the treatments containing extract, especially of reproductive stage, and the viability of the eggs was significantly influenced by the treatment with extract 1% of *E. pulcherrima* on reproductive stage, preventing 100% of the eggs from hatch (Table 4). Similar type of results were found by Alves et al. (2012), who has observed the low viability of *S. frugiperda* eggs from larvae fed diets containing methanolic extracts of *C. langsdorffii* leaves and bark of fruits. Silva et al. (2010) have found a reduction in the eggs viability from *S. frugiperda* larvae fed on artificial diet containing methanolic extract of *Piper hispidum* at concentrations 0.001, 0.006, 0.03, 0.2 and 1%. Santiago et al. (2008), using aqueous extract 10% of *Chenopodium ambrosioides* and *Licania rigida* on *S. frugiperda*, have found lower viability compared to the control treatment, indicating a possible negative effect on insect fertility. According to Costa et al. (2004), the viability of eggs and other parameters of fertility and fecundity are important effects of plant extracts on the reproduction of insects, which can be associated with eating disorders due to nutritional deficiency.

The study of the effects of plants with insecticidal properties should not aim only the mortality of insects, because for this purpose the amount required of the product is more than that used in this study making it practically an unviable technique. The main objective is that the plants have effects in reducing feed, fecundity and fertility, causing damage to future generations (Vendramim et al., 2000).

In this experiment, the extract to 1% of *E. pulcherrima* leaves collected in the reproductive phase, decreased the number of *S. frugiperda* eggs, and prevented them completely, proving its potential to efficiently reduce the populations of this particular insect in agricultural areas.

**Table 4.** Number of egg masses per couple (average), number of eggs per couple (average), and eggs viability (%) of *S. frugiperda* larvae fed on an artificial diet containing ethanolic extract of *E. pulcherrima* leaves.

Extract	Number of egg masses per couple (average)	Number of eggs per couple (average)	Viability (%)
TT	3.57±0.947 <sup>a</sup>	547±114.8 <sup>a</sup>	99.3±0.002 <sup>a</sup>
VE0.5%	2.28±0.837 <sup>a</sup>	303±159.6 <sup>a</sup>	98.5±0.005 <sup>a</sup>
VE1%	1.42±0.428 <sup>a</sup>	264±173.4 <sup>a</sup>	97.9±0.005 <sup>a</sup>
RE0.5%	1.28±0.521 <sup>a</sup>	106±54.40 <sup>a</sup>	97.7±0.007 <sup>a</sup>
RE1%	0.85±0.459 <sup>a</sup>	136±68.20 <sup>a</sup>	0 <sup>b</sup>
	P-value = 0.122258	P-value = 0.069288	P-value = 0.000

Means followed by the same letter in the column do not differ significantly by the Kruskal-Wallis test at 1%.

## Conclusions

- 1) The extract of vegetative and reproductive stages of *E. pulcherrima* leaves 0.5 and 1% concentrations has caused larval mortality of *S. frugiperda* up to 26%.
- 2) The larval period was prolonged by treatment with a vegetative extract 1% (19 days) and reproductive extract 0.5 and 1% (19 and 18 days respectively).
- 3) The larval weight was reduced by 40% with the reproductive extract at concentrations 0.5 and 1%.
- 4) Treatments with leaf extract of the reproductive phase (0.5 and 1%) reduced the weight of pupae.
- 5) The same extract above resulted in reduced rates of feces excreted by the larvae.
- 6) The leaf extract of the reproductive phase (1%) had as effects on *S. frugiperda* fertility, reduction of the number and decrease of the viability of the eggs.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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*Full Length Research Paper*

## Efficiency of the partial substitution of agar with potato starch on the growth and phytochemical parameters of lulo (*Solanum quitoense*) cultured *in vitro*

D. A. Martin G.<sup>1\*</sup>, Oswaldo E. Cárdenas-González<sup>1</sup>, José Pacheco<sup>2</sup>, Maritza De Jesus-Echevarría<sup>3</sup> and Jovanny A. Gómez Castaño<sup>1</sup>

<sup>1</sup>Laboratorio de Espectroscopia y Análisis Instrumental, Grupo Química–Física Molecular y Modelamiento Computacional (QUIMOL), Escuela de Ciencias Químicas, Facultad de Ciencias, Universidad Pedagógica y Tecnológica de Colombia (UPTC), Avenida Central del Norte, Tunja, Boyacá, Colombia.

<sup>2</sup>Grupo de Investigación BIOPLASMA-UPTC, Escuela de Ciencias Biológicas, Facultad de Ciencias, Universidad Pedagógica y Tecnológica de Colombia (UPTC), Avenida Central del Norte, Tunja, Boyacá, Colombia.

<sup>3</sup>Environmental Research Chemical Laboratory, Universidad de Puerto Rico, Recinto Universitario de Mayagüez, Mayagüez, Puerto Rico.

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The demand for agar, thanks to its different uses, generates the need to look for alternative substances that replace it. This work studied the influence of four different additions of potato starch into agar media (T1 = 50%, T2 = 45%, T3 = 40% and T4 = 0%) on the growth and chemical composition of essential oils of lulo (*Solanum quitoense*) explants cultured *in vitro*. The lengths, fresh and dry biomass weights, and number of nodes of the lulo explants were selected as growth indices and compared against either viscosity of the modified media or extraction percentage and relative concentration of the main components in the essential oils. A higher statistic variability and amount, of the lengths, fresh biomass and number of nodes, were found for the explants cultured in modified agar media compared to those cultured in pure agar. In contrast, minimal variation in metabolite extractions and concentrations were obtained as a function of potato starch addition. Cyclotetradecane, neophytadiene, 1-hexadecene, phytol, oleamide and 3-octadecene were found as the main components of the essential oils by gas chromatography-mass spectrometry (GC-MS). It was found that T1 was the treatment that yielded the best results in the analyzed variables.

**Key words:** *Solanum tuberosum* starch, *in vitro* culture, micropropagation, partial substitution, *Solanum quitoense in vitro* essential oil, secondary metabolites.

### INTRODUCTION

The current increase in the human population has been sustained due to the global expansion in agricultural production, provided mainly from the “green revolution”, which occurred during the second half of the last century, particularly in developing countries. Modern agriculture

requires pathogen-free seeds or propagules of high-quality that must be readily obtained. One of the more efficient and practical techniques presently used to produce seedlings is plant cell/tissue culture (Martin et al., 2013). However, due to the high-cost of the agar used

as gelling agent in the culture medium, this method is expensive and unsuitable for small farmers. The high demand for agar, to use for *in vitro* micropropagation, microbiological techniques and as an additive in various products, has led to the development of less expensive and more readily accessible substitutes (Jain and Babbar, 2002). In this regard, the use of gums and starches of natural origin has been shown to be a promising strategy to replace the commercial agars in culture medium (Ozel et al., 2008; Mohamed et al., 2010).

Starches are natural polysaccharides with many industrial uses (Pacheco and Techeira, 2009) because of their functional properties attributed to the variability of their amylose (linear polymer) and amylopectin (branched polymer) contents, which depend on the species and origin (Salinas et al., 2003). In particular, the outstanding thickening and binding properties of starch make it a suitable gelling agent and agar substitute in plant culture media. Indeed, cassava starch has been successfully used as a gelling agent for the micropropagation of *Celosia* spp. *Solanum lycopersicum* species (Daud et al., 2011a, b), *Musa* spp. (Mbanaso, 2008), *Faidherbia albida* and *Uapaca kirkiana* (Maliro and Lameck, 2004). Likewise, gelled starches from rice (González and Silva, 1999), corn (Zimmerman et al., 1995), sago (Rodríguez and Hechevarría, 2006) and enset (Mengesha et al., 2012) have been used for the *in vitro* propagation of species of *Theobroma cacao* L. and *Malus domestica* Borkh., *Orthosiphon aristatus* (Blume), *Artemisia absinthium* L., and *Vanilla planifolia*, respectively. In addition, some starch mixtures, such as agar/potato/corn (Mohamed et al., 2010) and flour/potato-semolina (Sharifi et al., 2011) have also shown to be good gelling agent in media for micropropagation of *Solanum tuberosum* and *Saintpaulia ionantha*, correspondingly.

Despite the diversity of starches evaluated as alternatives to commercial agar gelling agents, very little is known about the effect of using these types of modified media on the essential oil composition and metabolite concentration of the plants cultured *in vitro*. In this regard, Paek et al. (2005) stated that the most impact of the culture media in plant micropropagation should be observed on its primary and secondary metabolic routes. This was reinforced by the study of Pérez and Jiménez (2011), where the variations in the composition of secondary metabolites of micropropagated species were mainly attributed to the changes in the levels of growing regulators and carbon source and the concentrations of micro- and macronutrients available in the culture media.

In this context the objective of the work was to study the effect of partial agar substitutions with potato starch to culture media, on both the growth and the secondary

metabolites production of *in vitro* micropropagation of lulo (*S. quitoense* L.).

## MATERIALS AND METHODS

### Location of the study

The research was carried out in the instrumental analysis laboratory (Research Group QUIMOL), and the plant culture laboratory (Research Group BIOPLASMA). Results were acquired from June 2013 to March 2014. In this publication the data of a second subculture were taken, because in an earlier publication, information was published on some variables in the first subculture (Martin et al., 2013).

### Culture media

The base medium used for growing lulo nodal explants was MS (Murashige and Skoog, 1962), which was supplemented with 0.5 mg/L of indole-butyric acid and solidified with 7.5 g/L of agar (Difco). Four different agar/potato-starch mixtures (treatments), at ratios of 50/50 (T1), 55/45 (T2), 60/40 (T3) and 100/0 (T4), were prepared as culture media. All culture media were autoclaved at 121°C, 1 kg/cm<sup>2</sup> for 20 min.

The culture media viscosity was measured at 25°C, 10% torque from 0.3 to 3.0 rpm using a Brookfield LV DV-E viscometer equipped with an S-64 spindle.

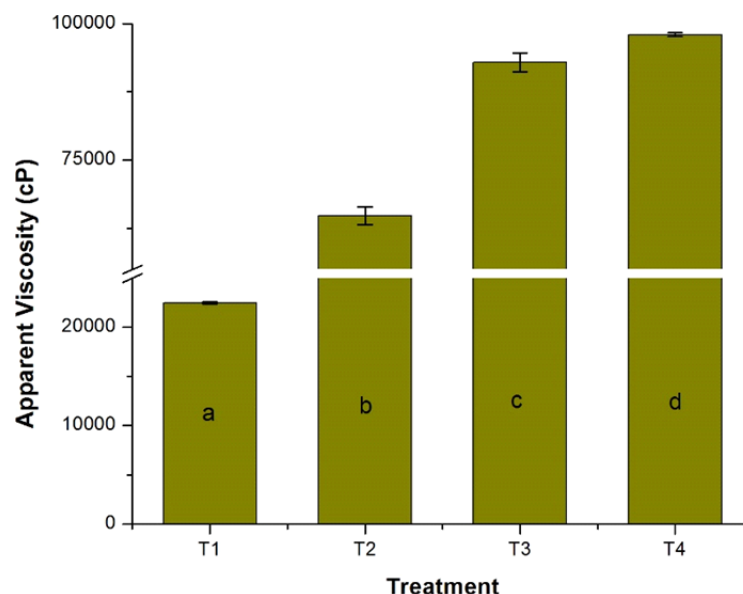
### Inoculation and incubation

Four nodal segments (approximately 2 cm in length) of lulo (*S. quitoense* L) were inoculated in 150 ml culture flasks containing 20 ml approximately of MS medium and illuminated at 25 ± 2°C and irradiated with a light intensity of approximately 100 μmol m<sup>-2</sup>s<sup>-1</sup>. For each of the four tested media, 72 explants were used for the first subculture and 192 for the second (45 days per subculture).

### Assessment of the effects of the partial replacement of agar

The number of nodes, seedling length, and fresh and dry biomass weights were selected as measures of plant growth. For each treatment (T<sub>n</sub>), a total of 0.63 to 0.79 g of dried plant material and 150 ml of solvent (dichloromethane) were used for Soxhlet-solid-liquid extraction of essential oil for 6 h. The extracts obtained, were concentrated in a rotary evaporator (Buchi, model R-205) using an internal pressure of 732 mbar. GC-MS analysis of the extracts was performed using a gas chromatograph (model 5890 series II, Hewlett Packard), equipped with a mass selective detector (MS 5972) and a Restek RTX-5 (30 m × 0.25 mm id × 0.25 μm) capillary column (crossbond 5% diphenyl/95% dimethyl-polysiloxane). The column temperature ramp was set at 70°C, for 4 min, then increased at 10°C/min up to 125°C, maintained at 125°C for 5 min, then increased at 2°C/min to reach 250°C, which was maintained for a further 12 min. The helium flow was 50 ml/min and the injection temperature was 225°C. The extract samples were diluted in 0.5 mL of chloroform and then an aliquot (0.5 μL) injected into the GC-MS using direct mode. Chemical identification of the extracts was performed by comparison of the GC-MS spectra with the MS

\*Corresponding author. E-mail: dario.martin@uptc.edu.co.



**Figure 1.** Apparent viscosity of the four agar/potato-starch mixtures in culture media of Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean  $\pm$  SD (n=3).

spectra stored in Wiley6.1 MS data library of natural products.

### Statistical analysis

Data analysis was performed using design experimental complete at random with four treatments (50/50 (T1), 55/45 (T2), 60/40 (T3) and 100/0 (T4) agar/starch ratios) of six replications each. The effects caused by the partial substitution of agar with potato starch were numerically analyzed using an ANOVA single factor treatment along with Duncan's multiple range test, with a significance level of 5% ( $p < 0.05$ ) in SPSS 17 version software. For the variables of explant length, node and leaf numbers, as well as fresh and dry weight, a mean of 32 explants was used as the sampling unit. Three replicates for each culture media treatment were obtained by splitting the plant dry material into three aliquots.

## RESULTS

### Viscosity media

The progressive partial substitution of agar with potato starch decreased the apparent viscosity of the culture media (T1, T2 and T3) compared to the starch-free culture medium (T4), as shown in Figure 1. However, in all instances, the consistency of the culture media remained stable enough to ensure, on one hand, the vertical position of the explants and, on the other hand, the conditions for the adequate development of seedlings, thus, allowing the plants to grow with both a normal root system as well as a physiologically active appearance (Figure 2).

It was observed that media consistency increased at

the end of the subculture, but it was neither measured nor analyzed.

### Growth indices

#### Seedling length

As depicted in Figure 3, the seedling lengths ranged from of 1.10 to 10.60 cm, corresponding to a significant difference in the ANOVA analysis ( $p = 0.000$ ). As also shown in Figure 3, the longer explant lengths were consistently derived from the agar culture media enriched with potato starch (T1 to T3).

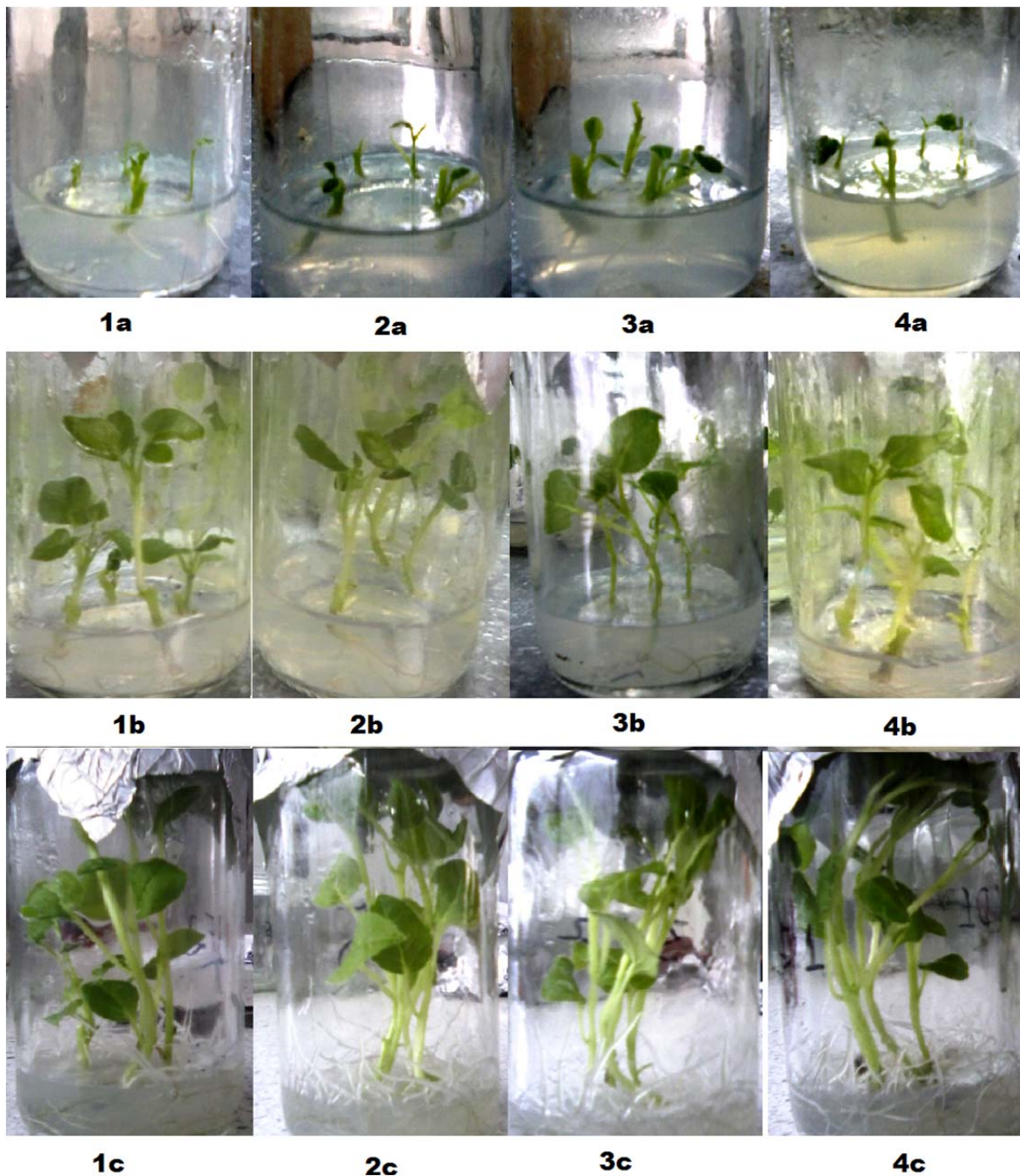
#### Number of nodes

As also shown in Figure 3, all explants grown in potato starch culture media (T1 to T3) had fewer nodes (1 to 9) compared to those grown in starch-free media (T4) ( $p = 0.09$ ).

#### Fresh and dry weights of biomass

The fresh and dry biomass weights ranged from 4.66 to 8.47 g ( $p = 0.036$ ) and 0.27 to 0.46 g ( $p = 0.268$ ) respectively. However, the lowest values were always obtained from the explants cultured in the medium free of potato starch (T4) (Figure 4).





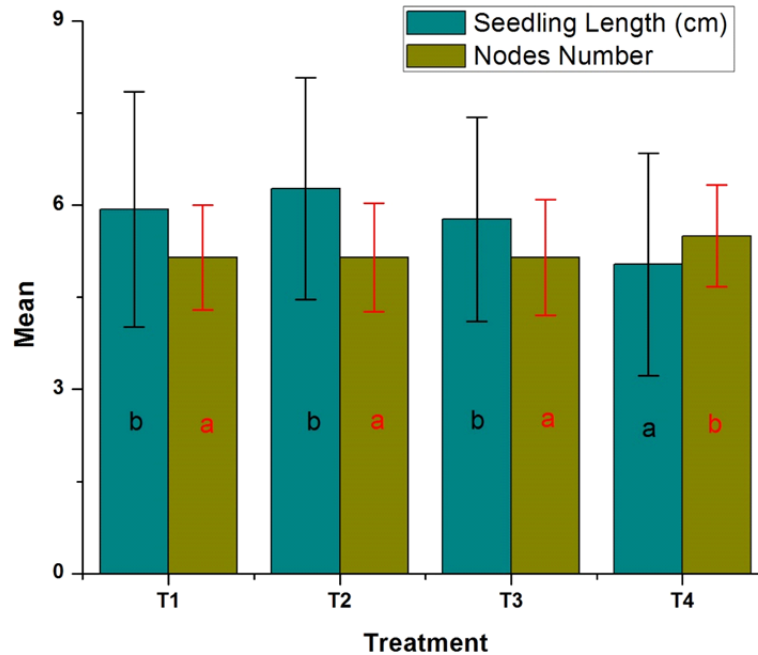
**Figure 2.** Physical appearance of the lulo (*S. quitoense*) explants in four treatments (T1 to T4) after 12 (a), 24 (b) and 40 (c) culture days.

### Essential oils

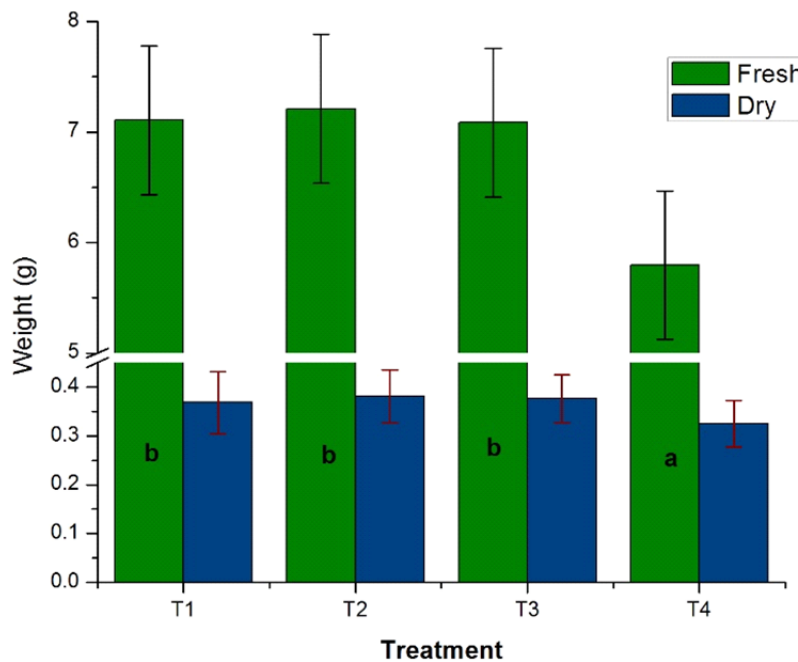
The percentages of essential oil extracted from the explants, ranged between 9.7 and 19.0% (dry biomass basis), as shown in Figure 5. Significantly less essential oil was produced by the explants grown in the culture media containing potato starch ( $p = 0.970$ ).

### GC-MS analysis of the essential oils

Table 1 lists the main molecular components in the essential oils extracted from the lulo explants cultured in modified potato-starch media *in vitro*, as detected by GC-MS. A comparison of the relative concentration means of the major components in the essential oils derived from



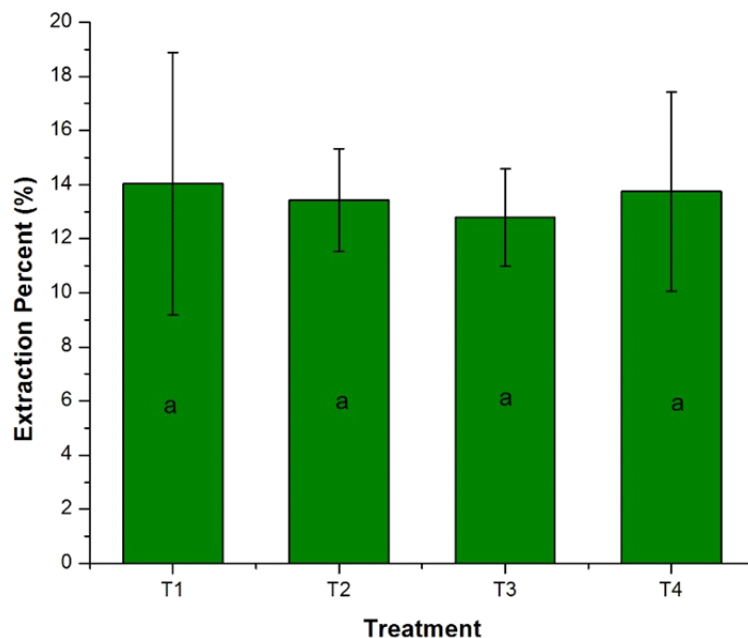
**Figure 3.** Mean seedling length and number of nodes for explants. Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean ± SD (n=6).



**Figure 4.** Biomass produced after 45 days of culture treatment. Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean ± SD (n=6).

the explants presented no significant difference, irrespective of the culture media: Dodecanol (p = 0.423),

cyclotetradecane (p = 0.768), neophytadiene (p = 0.470), 1-hexadecene (p = 0.442), phytol (p = 0.596), palmitic



**Figure 5.** Essential oil percentages extracted as a function of culture media modified with potato starch. Letter a not indicate statistical differences of 5% according to Duncan's test. Values are presented as mean  $\pm$  SD (n=3).

**Table 1.** Main molecular components in the essential oils of the lulo (*S. quitoense*) explants cultured in modified agar/potato-starch media. Values are presented as mean  $\pm$  SD (n=3).

Molecule	T1	T2	T3	T4
Dodecanol	8.26 $\pm$ 7.09	17.90 $\pm$ 5.08	12.21 $\pm$ 10.30	14.36 $\pm$ 2.15
Cyclotetradecane	6.17 $\pm$ 6.85	5.45 $\pm$ 4.88	5.87 $\pm$ 5.14	6.55 $\pm$ 0.83
Neophytadiene	1.13 $\pm$ 1.10	2.35 $\pm$ 3.26	11.20 $\pm$ 15.86	3.91 $\pm$ 1.55
1-Hexadecene	0.84 $\pm$ 1.19	2.87 $\pm$ 2.72	2.55 $\pm$ 2.25	3.37 $\pm$ 0.71
Phytol	0.51 $\pm$ 0.44	0.78 $\pm$ 0.71	0.51 $\pm$ 0.43	0.49 $\pm$ 0.15
Palmitic acid	1.89 $\pm$ 3.23	12.31 $\pm$ 3.36	0.03 $\pm$ 0.01	9.11 $\pm$ 15.75
Oleamide	7.28 $\pm$ 9.67	9.72 $\pm$ 8.57	13.81 $\pm$ 3.07	8.34 $\pm$ 14.40
3-Octadecene	2.78 $\pm$ 0.82	1.32 $\pm$ 1.15	1.52 $\pm$ 0.25	1.38 $\pm$ 1.30

acid ( $p = 0.273$ ), 3-octadecene ( $p = 0.283$ ) and oleamide ( $p = 0.855$ ). This indicated a negligible effect of the partial substitution of agar medium with potato starch in the formation and relative concentration of secondary metabolites of the lulo explants cultured *in vitro*. Other molecules, such as limonene, cresol, 1-octadecene, heneicosane and hexatriacontane were also detected in the essential oils of lulo explants but at lower amounts.

## DISCUSSION

The lower viscosity of the culture media containing potato starch can be attributed to the weaker gelling effect of the

starch compared to agar (González and Silva, 1999; Martin et al., 2012). The difference arises from the stronger interaction that the sulfate and pyruvate groups of agar form with water molecules, resulting in higher viscosity and consistency of the media, compared to the interaction between the hydroxyl moieties of starch and water molecules. A decrease in media consistency and solidification was also reported by Jain and Babbar (2011) and by Lucyszyn et al. (2006) in the partial substitution of agar with guar gum, isubgol or xanthan, and with galactomannans, respectively.

However, that lowers viscosity of the culture media showed positive effects on the plantlets growth. Thus the longer lengths of lulo explants cultured in enriched potato

starch media, can be associated with a higher absorption of nutrients (Sharifi et al., 2011), and the availability of specific nutrients, like maltose, saccharose and glucose, formed by hydrolysis of starch molecules during autoclaving (Maliro and Lameck, 2004). Explants with longer lengths have also been reported in cell and tissue culture e.g. in somatic embryogenesis of cultured in media enriched with isubgol (Ozel et al., 2008), micropropagation of cultured in gelling media containing mixtures of isubgol/agar and guar gum/agar (Jain and Babbar, 2011), micropropagation of *Dioscorea alata* and *D. trifida* using phytigel as cultured media (Chacón et al., 2000), and propagation of culture media gelled with cassava starch (Maliro and Lameck, 2004). A decrease in the length of explants cultured in modified agar media has been reported by Lucyszyn et al. (2005) for *in vitro* propagation of apple burgeon (*Malus prunifolia* Borkh.) using guar gum/agar and cassia gum/agar mixtures as gelling media at 50/50 ratio.

Similar to the present results, a decrease in the number of nodes has been also observed for other plant species cultured in modified media using natural gelling agents. Rodríguez and Hechavarría (2006) reported fewer leaves and nodes in *O. aristatus* and *A. absinthium* explants micropropagated using solidified media with either full or partial replacement of agar with sago flour and Aloe vera gel. As well, Mohamed et al. (2010) and Mengesha et al. (2012) found fewer nodes in explants of *S. tuberosum* and *V. planifolia* cultured in media enriched with mixtures of agar/potato starch (40-60%) and agar/enset starch (0.2%/6%), respectively, compared to starch-free media. In addition, both previous authors observed statistical differences among the various starch/agar ratios used, which is due not only to the particular nutritional requirements and metabolic behavior of each species cultured *in vitro* but also to stem elongations, which decrease the number of nodes.

In the present study, the increase in fresh biomass obtained from the lulo explants cultured in agar media partially substituted with potato starch compared to agar alone, reflects a higher absorption of micro- and/or macronutrients and water, increasing the cellular metabolism (Sharifi et al., 2011). As aforementioned, the availability of these nutrients is favored by the lower viscosity and consistency of media partially substituted with potato starch (Mbanaso, 2008). Mohamed et al. (2010) and Mengesha et al. (2012) also reported an increase in fresh biomass in potato nodal segments and *V. planifolia* explant micropropagated by culture media modified with mixtures of agar and corn, potato or enset starch, respectively. In contrast, Romay et al. (2006) showed no statistical differences in fresh material weight of cassava explants by replacing phytigel with cassava starch as culture media. The low variability in biomass weights contrasts with the length and number of nodes of the explants observed among the various media treatments, suggesting a minimal dependence of this

variable on the agar/potato starch media.

To date, the chemical composition of *S. quitoense* essential oils has been reported for the fruit (Silva et al., 1990; Acosta et al., 2009) and seeds (Jurado and Muñoz, 2009). However, no published reports are found regarding the composition of essential oil from this plant cultured *in vitro*. According to Silva et al. (1990), the fruit aroma of lulo *S. quitoense* is attributed mainly to the presence of methyl butyrate, ethyl acetate, 2-(E)-methyl butyrate, linalool,  $\gamma$ -hexalactone,  $\alpha$ -terpineol and acetic acid. Whereas the essential oil of lulo fruit are consisted of methyl benzoate, methyl hexanoate, 4-hydroxy-4-methyl-2-pentanone, butyric acid, methyl 3-hydroxyhexanoate, 2-methyl-2-pentenal and 2-(E)-methyl butanoate (Acosta et al., 2009). Conversely, Jurado and Muñoz (2009) reported high concentrations of palmitic, oleic and linoleic acids in essential oil extracted from seeds of *S. quitoense* variety.

Several of the compounds presently found in the essential oils (Table 1) have been also identified in other species belonging to the same genre. Such as for instance, 6.09% of 1-dodecanol detected in the essential oil of *S. sessiliflorum* fruit (Marx et al., 1998) and neophytadiene, phytol, 1-hexadecene and 1-octadecene in essential oils extracted from *S. subinerme* (Ordaz et al., 2011).

The low variability in the relative concentrations of the main compounds in the essential oil of lulo explants regarding culture media treatment indicates a negligible influence on the secondary metabolism of the plant for all ratios of potato starch added into the agar media. Therefore, variations in the relative concentrations of the main essential oil components for each treatment (Table 1) should be associated with specific physiologic or metabolic factors rather than absorption of potato starch by the plantlet. This type of association was inferred previously by Pérez and Jiménez (2011), who observed significant variations in the concentration of some secondary metabolites by the action of the culture media which should mostly originate from changes in growth regulators and the carbon source. In this context, Paek et al. (2005) corroborated that modifying the amounts of micro- and macronutrients could induce selective formation of metabolites in plants cultured *in vitro*.

## Conclusion

Significant differences in each of the analyzed variables show that the partial substitution of agar by potato starch produced positive effects in the growth and development indexes analyzed, probably because the decrease in the viscosity of these media allows a better diffusion of nutrients. In addition, the percentages of essential oil and the relative concentration of the major secondary metabolites were not significantly modified, allowing to recommend potato starch as an efficient partial substitute

as a solidifying agent for the culture media used for *in vitro* micropropagation of lulo (*S. quitoense*). It was found that T1 was the treatment that yielded the best results in the analyzed variables.

### Conflict of Interests

The authors have not declared any conflict of interests.

### ACKNOWLEDGEMENTS

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*Full Length Research Paper*

# Evaluation and association mapping of agronomic traits for drought tolerance in sorghum [*Sorghum bicolor* (L.) Moench]

Aleye Endre<sup>1\*</sup> and Kassahun Bante<sup>2</sup>

<sup>1</sup>Amhara National Regional Agricultural Beureau, Dessie Soil Testing Laboratory, Dessie, Ethiopia.

<sup>2</sup>Jimma University, Department of Plant Sciences, Institute of Biotechnology, Jimma, Ethiopia.

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Drought is the major sorghum production constraint in Ethiopia which necessitates the identification of sorghum genotypes that carry genes (quantitative trait locus, QTL) associated with drought tolerance thereby developing drought tolerant sorghum varieties. The objectives of this study were to identify drought tolerant sorghum genotypes, map chromosomal regions (QTLs) associated with agronomically important traits and identify simple sequence repeat (SSR) markers tightly linked with these QTLs. One hundred and sixty (160) sorghum genotypes (152 landraces and 8 released varieties) were genotyped with 39 SSRs markers and evaluated in the field at Kobo in the off-season using an alpha lattice design replicated three times. Phenotypic data including days to 50% flowering, plant height, panicle weight, grain weight, grain weight per panicle, panicle harvest index, one thousand grain weight and number of grains per panicle were collected. Analysis of variance showed highly significant ( $P < 0.0001$ ) differences among the genotypes for all characters. Most of the characters showed moderate to high phenotypic and genotypic coefficient of variation. Linkage disequilibrium (LD) analysis indicated that in all accessions, 107 loci pairs (32.92%) had a significant ( $p < 0.05$ ) mean LD of 0.19, with  $R^2 > 0.2$  for 33 evaluated loci pairs. Population structure analysis showed that there were four distinct clusters in the studied materials. A total of 10 marker-trait associations were identified using seven different SSR markers. The percentage of the total variation explained by the markers ranged from 2.6% (Xtxp114 with THGT) to 17.76% (Xtxp145 with PHT). The seven SSR markers (xcup53, bSbCIR223, Xtxp114, mSbCIR248, Xtxp145, Xtxp278, and gbsp123) were located on chromosomes 1, 2, 3, 5, 6, 7 and 8, respectively, each chromosome harboring one marker. Most of the identified markers were localized in chromosomal positions that have been previously reported as positions for drought tolerance-related traits, supporting the present findings. The results of this study can serve as initial effort for the association mapping studies in sorghum particularly in Ethiopia as the associated SSR markers are potential candidates for marker-assisted selection to improve drought tolerance in sorghum. However, as this study is the first attempt in the identification of QTLs for drought tolerance using association mapping, the identified QTLs need to be validated in independent or related populations and in different environments before their use in marker-assisted selection.

**Key words:** Association mapping, drought, population structure, quantitative trait locus (QTLs), sorghum, simple sequence repeat (SSR).

## INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is a largely self-pollinating (70-95%) monocot crop in the grass family of Poaceae with a diploid set of chromosomes ( $2n=2x=20$ ) and an estimated genome size of 750 Mb (Doggett, 1976; Yonemaru et al., 2009). It serves as a staple food for the world's most food insecure people, particularly in the semi-arid tropics of Asia and Africa. More than 35% of world's sorghum production is dedicated to human consumption of which 95% is in developing countries; the rest being used mainly for animal feed, alcohol and industrial products (Dicko et al., 2006). Worldwide, sorghum ranked fifth in production after maize, wheat, rice and barley (FAOSTAT, 2010). In Ethiopia, it ranked third after maize and tef with a total production of 2.8 million metric tons and an average yield of 1,736 kg/ha (FAOSTAT, 2010).

Despite its importance, sorghum productivity is severely limited by drought accounting for more than 50% yield losses each year globally (Hao et al., 2011). The severity increases particularly in developing countries like Ethiopia, where the majority of the people depend on agriculture for their livelihood (Hao et al., 2011). In Ethiopia, sorghum is largely cultivated in moisture stress areas that cover nearly 66% of the total area of the country (Tadesse et al., 2008). This necessitates evaluation and identification of genomic regions that confer resistance to drought stress particularly at the reproductive stage thereby developing drought tolerant varieties. Sorghum is naturally moisture stress tolerant crop which can be more productive with moderate genetic improvement. In sorghum, two distinct drought responses are recognized, pre-flowering and post-flowering drought responses (Sanchez et al., 2002). The pre-flowering response occurs when the plants are under significant moisture stress before flowering (Tuinstra et al., 1996). Post-flowering drought response in sorghum is expressed when moisture stress occurs during the grain development stage (Tuinstra et al., 1996). Drought at any stage of crop development affects growth and production but drought during the flowering stage causes maximum crop damage (Ejeta and Knoll, 2007). Under water-limited environments, genetic improvement of crops for drought tolerance is a sustainable and economically feasible solution to reduce the impact (Tadesse et al., 2008). However, in quantitatively inherited traits such as drought tolerance that are controlled by many genes each with small effects (QTLs), selection by conventional methods based on phenotypic variations is inefficient and challenging due to the complex nature of the trait and the complicating effects of the environment. Through QTL

mapping, marker assisted selection (MAS) using DNA markers has become more efficient to tag such traits (Hausmann et al., 2002). The most common approach of QTL mapping is to identify QTLs in a bi-parental population (Shehzad et al., 2009). Another approach being applied is association mapping, which uses diverse populations to identify associations between allele frequencies and phenotypic variations (Sorkheh et al., 2008). Unlike QTL mapping where bi-parental crosses with contrasting genotypes are used to generate a mapping population, association mapping is an approach where a collection of cultivars, lines, and/or landraces, genotyped with densely spaced markers, can be used as mapping population (Sorkheh et al., 2008; Myles et al., 2009). Using a collection of cultivars has a number of advantages over the use of a bi-parental cross. Firstly, in the population, a broader genetic variation in a more representative genetic background will be available implying that one is not limited to the marker and trait loci that happen to differ between two parents. Secondly, LD mapping can attain a higher resolution because of the use of all meioses (recombinations) accumulated in the breeding (selection) history. Thirdly, historic phenotypic data on cultivars can be used to link markers to traits, without the need to develop bi-parental mapping populations (Sorkheh et al., 2008). Thus, originally developed for human genetics, association mapping strategy now is being exploited in several crop plants. In sorghum, Shehzad et al. (2009) reported a total of 14 significant SSR loci associated with traits including days to heading, days to flowering, number of panicles and panicle length using 107 representative sorghum accessions and 98 SSR markers. Wang et al. (2012) reported two SSR markers consistently associated with plant height at two environments. Upadhyaya et al. (2012) reported significant associations of five markers with maturity date and plant height on chromosomes 6, 9, and 10 using 242 sorghum accessions and 39 SSR markers. These works emphasize the possibility of genetic improvement in sorghum using the existing germplasm resources (including land races) by evaluating in field; and identifying and mapping QTLs associated with desired traits and selecting the genotypes (parents) that carry favorable alleles for gene introgression through marker assisted selection. Ethiopia is rich in sorghum landraces as sources of desirable genes to screen genotypes for better agronomic performance under moisture stress (Amsalu et al., 2000). However, no study to detect marker-trait association for moisture stress tolerance in sorghum have been previously reported in Ethiopia using association mapping strategy which initiated the present study.

\*Corresponding author. E-mail: aleye.endre@gmail.com.

## MATERIALS AND METHODS

### Phenotyping

#### *The study area, plant materials and experimental design*

The field experiment was conducted in Ethiopia at Kobo agricultural research site. Kobo is located 581 km north of Addis Ababa, at an altitude of 1468 masl. (12°9' N latitude and 39°38'E longitude). The entries for this study consisted of 160 sorghum (*S. bicolor*) accessions (152 land races and 8 released lines) which were sampled from more than 1800 sorghum accessions collected by the Institute of Biodiversity Conservation (IBC) of Ethiopia. The samples were selected on the basis of their geographic distribution from all regions of the country representing sorghum growing areas with altitude range of 500 to 1850; more accessions from areas predominantly affected by moisture stress (regions from which the accessions were collected are shown in Supplementary Table 1). The seeds of released varieties were provided by Melkasa and Sirinka Agricultural Research Centers. These experimental materials were grown at Kobo during the off season of 2011 using irrigation from January to June (Kobo is one of the best sites in the country for sorghum variety performance trial, particularly, for drought tolerance as the site is located in the area where sorghum is predominantly grown but frequently affected by moisture stress). Mean monthly temperature and rain fall at the experimental site during the cropping period is shown in Supplementary Table 2. The field experiment was laid down in alpha lattice design with three replications having 16 blocks per replication and 10 plots per block and a spacing of 75 cm and 20 cm between rows and plants, respectively. Forty (40) plants were planted per plot in two rows of 4 m long. The experimental plots were irrigated immediately after sowing to ensure uniform germination. Weekly interval irrigation was applied for the first three weeks. Starting from the fourth irrigation, water was supplied with 12 days interval till 50% flowering (Tuinstra et al., 1998; Xu et al., 2000). Irrigation was withheld when the majority of the entries reached 50% flowering creating a terminal water deficit which typifies the dry season of the semiarid tropics, where crops are usually grown on a depleted soil moisture profile. The recommended fertilizer rate of 100 kg Di ammonium phosphate (DAP) was applied by incorporating it into the soil during sowing the seeds followed by 25 kg urea ha<sup>-1</sup> by side dressing 55 days after the seeds were sown. Thinning was conducted after three weeks of sowing to maintain the plant distance and to balance the plant density. Karate was applied two times with a rate of 1 mm<sup>-1</sup> of water 30 and 45 days after emergence to protect against shoot fly. No herbicide was applied to control weeds. Bird damage was protected by covering the heads of 10 randomly chosen plants from each plot to obtain the average grain yield per panicle. Other agronomic practices such as weeding were applied uniformly to all plots according to the recommended practices. Phenotypic data were collected for seven traits (50%FL-days to 50% flowering, PHT-plant height, PWT-panicle weight, GWPP-grain weight per panicle, THGT-thousand seed weight, NGPP-number of seeds per panicle and PHI-Panicle harvest index) based on sorghum descriptor list (IBPGR and ICRISAT, 1993).

### Genotyping

#### *DNA extraction*

The 160 accessions were grown in greenhouse and the fresh leaves of 10 plants from 14 days old seedlings were harvested in bulk and dried with silica gel. DNA was extracted following a modified cetyl trimethyl ammonium bromide (CTAB) extraction

protocol (Mace et al., 2003).

The quality and quantity of the isolated DNA was determined by comparing DNA samples with a known concentration of  $\lambda$ -DNA after running them on a 0.8% agarose gel (0.8 gm agarose dissolved in 100 ml 1X TBE buffer) that contained 0.3  $\mu$ g/ml ethidium bromide solution. At the end of electrophoresis, the gel was visualized using ultraviolet (UV) light and photographed using a video capture (Flowgen IS 1000). All samples were then normalized to the same concentration level (50 ng) and used for PCR.

### SSR markers used

A total of 39 simple sequence repeat (SSRs) markers, including 22 di, 9 tri, and 4 tetra nucleotide or longer motifs, and 4 compound repeats were used. These SSR markers were selected based on their uniform distribution in the sorghum genome. Four of them from chromosome SBI-01, five of them from chromosome SBI-02, four of them from chromosome SBI-03, two of them from chromosome SBI-04, four of them from chromosome SBI-05 and chromosome SBI-06 each, five of them from chromosome SBI-07 and chromosome SBI-08 each and three of them from chromosome SBI-09 and chromosome SBI-10 each. These are the same set of markers that are selected and being used by the Generation Challenge Programme for genetic diversity assessment of global sorghum germplasm.

### Polymerase chain reaction (PCR)

The PCR was performed in Nairobi (Kenya) using Gene-Amp PCR System 9600 (PE-Applied Biosystems) in 384-wells plates (ABGene, Rochester, New York.) in a total reaction volume of 10  $\mu$ l that consisted of 1  $\mu$ l DNA (50 ng), 1  $\mu$ l 10X PCR buffer, 1.5  $\mu$ l MgCl<sub>2</sub> (10 mM), 1  $\mu$ l reverse primer (2 pmoles), 1  $\mu$ l forward primer (2 pmoles), which were 5'-labelled with one of the 6-FAM, VIC, NED, PET fluorescent dyes (PE-Applied Biosystems), 0.5  $\mu$ l of each dNTP (2 mM), 0.04  $\mu$ l Taq DNA polymerase (5U) (PE-Applied Biosystems) and 3.46  $\mu$ l distilled water. The amplification profile consisted of initial denaturation of the template DNA at 95°C for 3 min, followed by 35 cycles, each for 30 s at 95°C (denaturation), 1 min at 56°C (annealing), and 1 min at 72°C (extension), and a final extension at 72°C for 30 min was included to minimise the +A overhang.

### Capillary electrophoresis

After the PCR, a few samples from each primer pair product were randomly selected and checked for proper amplification by comparing DNA samples with a known molecular weight of  $\lambda$ -DNA after running them on 2% agarose gel. An ABI plate was prepared with a total volume of 10  $\mu$ l (9.0  $\mu$ l from a mix of an injection solution mixed by vortexing (1 ml) formamide (HIDI) (Perkin Elmer-Applied Biosystems) and 12.0  $\mu$ l GS500 LIZ (Perkin Elmer-Applied Biosystems) was aliquoted into 96-well plates and 1.0  $\mu$ l of pooled PCR products from each of the 6-FAM, VIC, NED and PET-labelled PCR products was added. DNA fragments were denatured at 95°C for 3 min, chilled quickly for five minutes and size-fractionated using ABI 3730 Capillary DNA sequencer (PE-Applied Biosystems). In this system, the labeled PCR products were detected using a laser and capillary electrophoresis based on their fluorescent dye and fragment size. The peaks were sized and the alleles called using Gene Mapper software version 3.7 (PE-Applied Biosystems) and presented as alleles scored as estimated fragment sizes in base pairs compared to the internal size standard GS500LIZ-3730.

## Data analysis

### Phenotypic data

All collected phenotypic data were subjected to one way ANOVA using SAS software version 9.2 (SAS Institute Inc., 2008) for variances and heritability.

### Molecular data analysis

#### Linkage disequilibrium analysis

LD ( $R^2$ ) between SSR markers /loci/ was evaluated using Tassel software version 2.0.1. The LD was calculated using the statistical coefficient of determination ( $R^2$ ) (Shi et al., 2010). Alleles with frequencies less than 0.05 were not included for LD calculation.

#### Population structure (Q-matrix) and kinship (K-matrix) analysis

Population structure (Q-matrix) among 160 sorghum accessions was analyzed using 39 SSR markers by *STRUCTURE* software version 2.3.3 (Pritchard et al., 2000). By setting the number of k levels (number of sub groups) from 1 to 9 with five times repetition for each k, nine independent structure runs were performed with 100,000 burn-in time and 100,000 iterations for each run. All *STRUCTURE* runs were performed using the admixture model with the option of correlated allele frequency. Also, the measure of the degree of admixture, alpha, was allowed to be inferred from the data (Pritchard et al., 2000), and Lambda, the parameter of the distribution of allelic frequencies, was set to one. The matrix of kinship coefficient comparing all pairs of the 160 lines using 39 SSR markers was calculated by the software package SPAGeDi as described by Loiselle et al. (1995). Negative kinship values between two individuals, indicating that there was less relationship than that expected between two random individuals, were changed to 0 and the diagonal was set to 2 (Pritchard et al., 2000).

#### Analysis of marker trait association

By fitting the population structure and kinship matrix into the model to avoid spurious associations, the trait marker association was evaluated using a mixed linear model (MLM) in "TASSEL" software version 3.0.1. To achieve linear independence, the structure matrix (Q-matrix) with one column less than the number of sub populations was used (Pritchard et al., 2000). The statistical model used for identifying SSR markers associated with traits was as follows:

$$Y_{klmnf} = \mu + k + ML + Am(ML)Kn + \varepsilon_{kmn}$$

Where,  $Y_{klmnf}$  is the phenotypic observation,  $\mu$  is the general mean,  $k$  is the fixed effect of  $k^{th}$  subgroup of the population structure (Q-matrix),  $ML$  is the fixed effect of  $L^{th}$  marker,  $Am(ML)Kn$  is the random effect of  $m^{th}$  accession nested in the  $L^{th}$  marker associated with  $n^{th}$  kinship coefficient, and  $\varepsilon_{kmn}$  is the error. Only markers with an allele frequency  $\geq 5\%$  were included in the association analysis. The significance of associations between loci and traits was based on an F-test with P values calculated by TASSEL at 5% significant level (Wang et al., 2012).

## RESULTS AND DISCUSSION

The analysis of variance (ANOVA) for the studied characters showed that there was a highly significant ( $P <$

0.0001) difference among genotypes for all characters indicating wide variability in performance among the genotypes. Most of the characters showed moderate to high phenotypic and genotypic coefficient of variation. High heritability coupled with high genetic advance as percentage of mean is the most promising clue for possibility of improvement by selection and was observed for PHT, PWT, GWPP, THGT and NGPP.

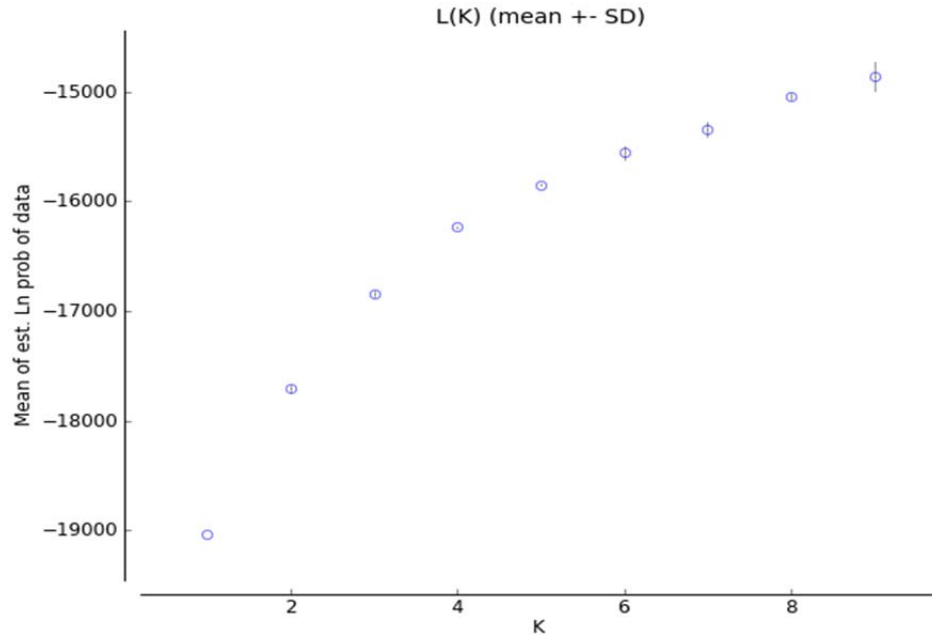
### Population structure

The population structure analysis showed that the 160 sorghum accessions contained four distinct sub groups (Figures 2 and 3). Actually, the plot of the average log likelihood values over five runs for each K (ranging the k-values from 1 to 9) showed that the log likelihood estimates increase progressively as K increases (Figure 1) and did not show a clear peak to determine the true K (number of sub groups). To reliably detect the most probable number of sub-populations, the ad hoc criterion described by Evanno et al. (2005) was used and the number of sub-populations were found to be 4 (Figure 2) which was selected and used for association analysis. Plots of ancestry estimates provided the estimated membership coefficients for each individual in each cluster. Each sorghum variety is represented by a single vertical line, partitioned into K colored segments that represent individual varieties estimated membership fraction in each of the K inferred clusters (Figure 3). The population structure analysis also indicated that sorghum accessions were not clustered according to their areas of collections; rather in each cluster were sorghum varieties from different areas of collections. For example, the first group, G-1 (Figure 3) consisted of 52 accessions of which 20 were from Amhara, 16 from Oromia, 9 from Tigray, 1 from Afar and other 5 accessions which their geographical origin was not available. Similarly in the 2<sup>nd</sup> (G-2), 3<sup>rd</sup> (G-3) and 4<sup>th</sup> (G-4) groups' clusters were composed of accessions from different areas of collections.

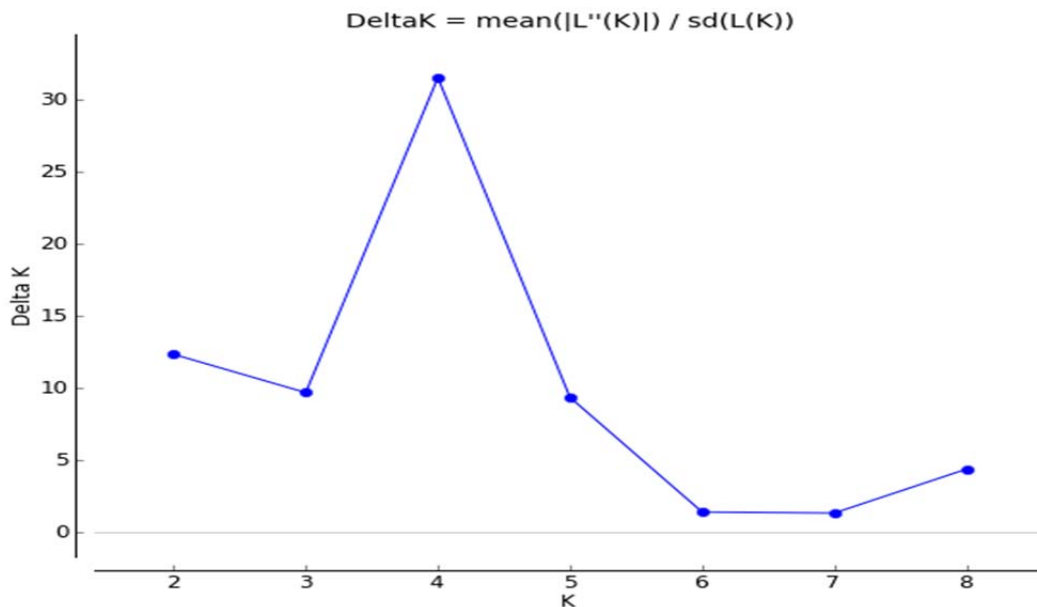
The distribution of accessions into the four groups without reflecting their region of origin might indicate the presence of wide variations among accessions within the regions as well as lack of strong regional differentiation which might be due to gene flow between the regions. Similar results that showed lack of clustering based on the collection sites of sorghum accessions were reported by Alemu (2009).

### Level of linkage disequilibrium

In this study, all 39 SSR markers were used to estimate the presence of LD in all accessions. After filtration of the data to exclude markers with less than 5% allele frequencies from the analysis, there were 325 pair wise locus comparisons for all accessions and the majority of



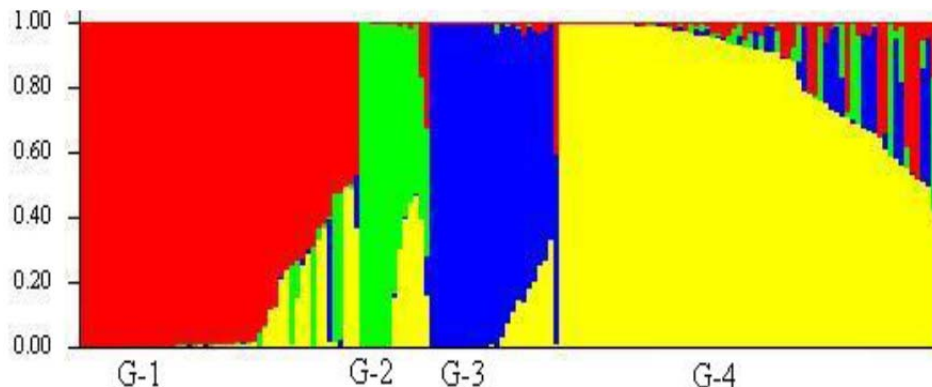
**Figure 1.** Posterior probability,  $\ln P(D)$ , of the data as a function of the number of subpopulations ( $k$ ), where  $k$  was allowed to range from 1 to 9. Circles represent the average of the five independent runs for each value of  $k$ .



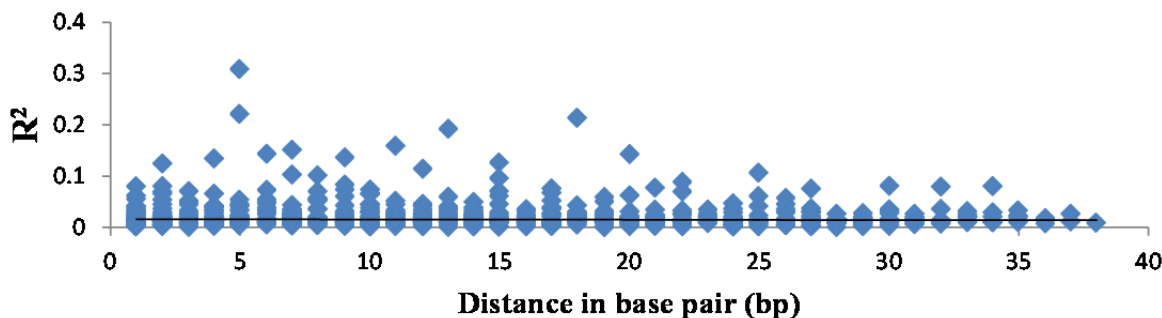
**Figure 2.** Values of  $K$  (x-axis), with its modal value used to detect the true  $K$  (y-axis) of four groups ( $K = 4$ ).

loci pairs (67.077%) were independent loci (non-significant). In all accessions, 107 loci pairs (32.92%) had a significant ( $p < 0.05$ ) mean LD of 0.19, with an  $R^2 > 0.2$  for 33 evaluated loci pairs. However, the present study did not show a clear trend on linkage disequilibrium

decay (Figure 4) and no clear conclusions can be made regarding the decay of LD. This result might be explained by low number of markers used in this study. Similar results were reported by Shehzad et al. (2009) using 107 sorghum accessions and 98 SSR markers and Li et al.



**Figure 3.** Population structure in the studied entries. The subpopulations obtained with K= 4 are represented by different colors as indicated at the bottom (G-1=red, G-2=green, G-3=Blue, and G-4 = yellow).



**Figure 4.** Linkage disequilibrium decay plot generated by 39 SSR markers.

**Table 1.** Associations between SSR markers and six agronomical traits.

Trait	Marker	Chr.	Position (Mb)	F-value	p-value	Marker R2
GWPP	gpsb123	C8	52.282	5.34565	0.0236	0.07626
PWT	gpsb123	C8	52.282	4.46473	0.03802	0.06574
PHI	gpsb123	C8	52.282	4.4633	0.03805	0.04638
PHT	mSbCIR223	C2	4.657	4.23342	0.04232	0.04077
PHT	mSbCIR248	C5	4.746	5.79222	0.01803	0.05389
THGT	Xcup53	C1	72.905	4.22234	0.04333	0.03798
THGT	Xtxp114	C3	60.794	4.72867	0.0313	0.02642
PHT	Xtxp145	C6	49.285	10.4089	0.0021	0.17757
50%FL	Xtxp145	C6	49.285	4.12649	0.04697	0.08347
50%FL	Xtxp278	C7	51.120	4.14561	0.04426	0.04183

GWPP=grain weight per panicle, PWT=panicle weight, PHI=panicle harvest index, PHT=plant height, THGT=thousands grain weight, 50%FL=Days to 50% flowering. Only SSR markers with a significant marker-trait associations are reported ( $P < 0.05$ ). The P-value determines whether a QTL is associated with a marker, and the marker R2 evaluates the magnitude of the QTL effects (percentage of total variation explained by the marker).

(2010) using 26 sorghum inbred lines.

**Association mapping**

In this study, a total of 10 significant marker-trait

associations ( $P \leq 0.05$ ) were detected (Table 1) and the phenotypic effect of SSR marker alleles on the associated characters were identified (Table 2). The 10 significant marker-trait associations were identified using seven different SSR markers for six agronomical characters (50%FI, PHT, PWT, GWPP, THGT and PHI),

**Table 2.** The phenotypic effect of marker alleles at loci associated with traits and the number of accessions carrying each marker allele in the studied sorghum accessions.

Character	Marker	Chr.	Pos(Mb)	Genotype (bp)	Effect	Observation
50%FL	Xtxp145	C6	49.285	214:214	-7.1919	46
50%FL	Xtxp145	C6	49.285	212:212	0	15
50%FL	Xtxp278	C7	51.12	248:248	4.92614	62
50%FL	Xtxp278	C7	51.12	242:248	0	48
GWPP	gpsb123	C8	52.282	290:290	-15.866	44
GWPP	gpsb123	C8	52.282	292:292	0	34
PHI	gpsb123	C8	52.282	290:290	-0.063	44
PHI	gpsb123	C8	52.282	292:292	0	34
PHT	mSbCIR223	C2	4.657	105:111	0.17516	40
PHT	mSbCIR223	C2	4.657	105:105	0	62
PHT	mSbCIR248	C5	4.746	91:91	-0.2505	54
PHT	mSbCIR248	C5	4.746	101:101	0	46
PHT	Xtxp145	C6	49.285	214:214	-0.3974	46
PHT	Xtxp145	C6	49.285	212:212	0	15
PWT	gpsb123	C8	52.282	290:290	-17.307	44
PWT	gpsb123	C8	52.282	292:292	0	34
THGT	Xcup53	C1	72.905	182:182	3.5836	33
THGT	Xcup53	C1	72.905	182:186	0	48
THGT	Xtxp114	C3	60.794	231:231	-2.3798	37
THGT	Xtxp114	C3	60.794	233:233	0	112

50%FL = Days to 50% flowering, PHT = plant height, PWT = panicle weight, GWPP = grain weight per panicle, PHI = panicle harvest index, and THGT = thousands grain weight.

with  $R^2$  ranging from 2.6% (Xtxp114 with THGT) to 17.76% (Xtxp145 with PHT) given subsequently.

### **Days to 50% flowering**

Two SSR markers (Xtxp145 & Xtxp278) having a significant association ( $P \leq 0.05$ ) with days to 50% flowering were detected on chromosome 6 and 7, respectively. Xtxp145 had an effect of explaining 8.35% of the total phenotypic variation, whereas Xtxp278 had an effect of 4.18% of the total phenotypic variation. SSR markers linked to QTLs that control flowering time in sorghum were previously reported on chromosome six by Mannai et al. (2011) using association mapping and on chromosome 7 by Sirinivas et al. (2009) and Shiringani et al. (2010) using conventional QTL mapping.

### **Plant height**

Three loci (mSbCIR223, mSbCIR248, Xtxp145) having a significant association ( $P \leq 0.05$ ) with PHT were detected on chromosome 2, 5 and 6, respectively. Marker mSbCIR223 had an effect of 4.1% of the total phenotypic variation; mSbCIR248 had an effect of 5.39% of the total phenotypic variation, whereas Xtxp145 had an effect of

17.76% of the total phenotypic variation. Wang et al. (2012), using pool based genome wide association mapping, reported four SSR markers that were closely associated with PHT on chromosomes 2 and 6. Similarly, Sirinivas et al. (2009), using conventional method confirmed the presence of QTLs for PHT on chromosome 6 and 7 in sorghum.

### **Panicle weight, grain weight per panicle, and panicle harvest index**

Locus gpsb123 showed simultaneous significant associations ( $P \leq 0.05$ ) with three characters, namely PWT, GWPP, and PHI on chromosome 8. This locus had an effect of explaining 6.6, 7.6, and 4.64% of the total phenotypic variation for PWT, GWPP, and PHI, respectively.

### **Thousand grain weight**

Two loci (Xcup53 and Xtxp114) on chromosomes 1 and 3, respectively, showed significant association ( $P \leq 0.05$ ) with THGT. Xcup53 had an effect of 3.8% of the total phenotypic variation, whereas Xtxp114 had an effect of explaining 2.64% of the total phenotypic variation. A QTL



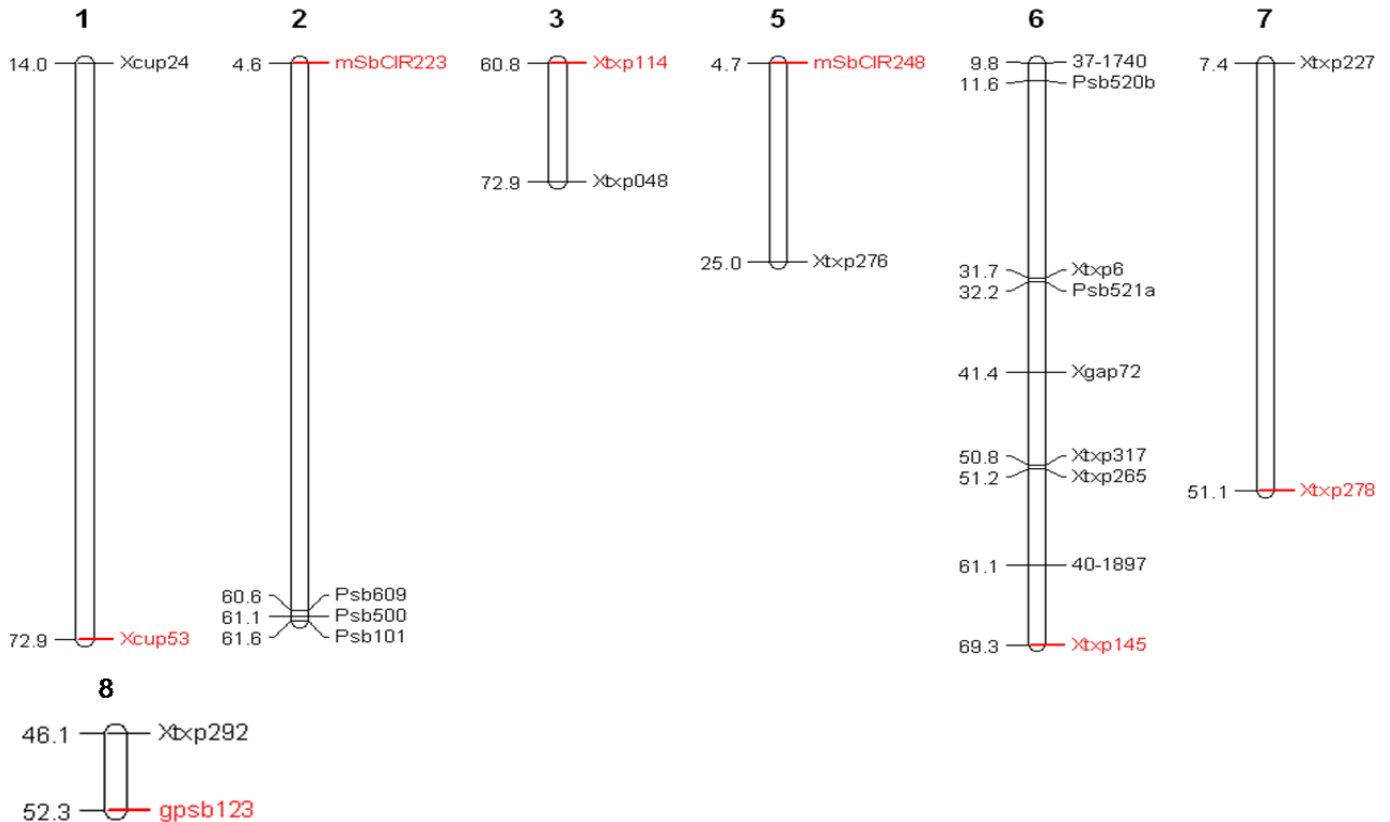
controlling seed weight was previously reported on chromosome one by Sirinivas et al. (2009) using conventional QTL mapping.

The present study also shows that in each of the identified SSR marker loci there were two genotypes having variant alleles in the studied accessions (Table 2). Most of the two genotypes at each locus had different magnitudes on the expression of the phenotype. As shown in Table 2, for example, for Xcup53 on chromosome 1, there were two genotypes (182:182 and 182:186) which were significantly associated with THGT. The presence of allele 182 in its homozygous state (182:182) increased the weight of thousand seeds by 3.5836 g compared to its heterozygous state (182:186). Similarly, for Xtxp114 on chromosome 3 which also linked to THGT, there were two genotypes (231:231 and 233:233). For this trait (THGT), the difference between the two genotypes (231:231 and 233:233) was 2.37984. In the same way, Xtxp145 on chromosome 6 which associated simultaneously with two traits: 50%FL and PHT, had two homozygous genotypes (214:214 and 212:212). The presence of marker allele 214 in its homozygous form decreased the days to 50% FL in 46 lines by 7.19 and the PHT by 0.39742 compared to its variant allele (212:212) in 15 accessions. Similarly, Xtxp278 on chromosome 7 which was found to be significantly linked to days to 50%FL had two genotypes (248:248 and 242:248) for the studied accessions. The presence of marker allele (248:248) increased the days to 50% FL by 4.92614 days compared to its variant allele (242:248). In the same way, gbsp123 on chromosome-8, which was found to be significantly associated with PWT and GWPP, had two genotypes (290:290 and 292:292). The presence of allele 290 in its homozygous form on this locus decreased PWT and GWPP by 17.307 and 15.866, respectively in 44 accessions compared to its homozygous variant allele (292) in 34 accessions for both traits. The difference of the effect on the phenotype between the two genotypes of mSbCIR223 on chromosome 2, mSbCIR248 on chromosome 5, and Xtxp145 on chromosome 6 which were found to be linked to PHT and between the two genotypes of gbsp123 on chromosome 8 to PHI, was negligible. Actually, it is not the variant marker allele itself which causes the decrease or increase in the expression of the phenotype in any trait of interest. Rather, it is imagined that there is a causative gene that is tightly linked to the variant marker allele which is responsible to cause a decrease or an increase in the expression of the phenotype. Thus, by following the variant marker allele that is tightly linked to the causative gene, it is possible to follow the effect of the causative gene on the phenotype of the lines under study. Generally, most of the variant alleles on the identified SSR marker loci had differences in magnitude of their effects on the phenotype of the trait under study. Some of the variant alleles had an increasing effect on the expression of the phenotype while others had a reducing

effect, this phenomenon might have useful application in molecular breeding. For example, if an interest arises to develop a variety of a grain cereal having a relatively short stature and earliness in flowering, a statistically linked marker allele with a reducing effect on plant height and days to 50% flowering shall be the target of the breeder. On the other hand, if the interest is to develop a variety for green forage having a relatively taller plant height and late flowering with high biomass accumulation, the marker allele with an increasing effect on plant height and days to 50% flowering shall be the target allele as plants with taller plant height and late flowering tend to accumulate high biomass for the purpose of green forage than with short stature and earliness in flowering (Habyarimana et al., 2004).

In sorghum, several reports have been published using conventional QTL mapping and some of them are in agreement with the present study. For example, in the study of Srinivas et al. (2009), one major QTL and two other QTLs were detected on chromosome 1 controlling seed weight which is similar to the present finding where locus Xcup53 on chromosome 1 is found to be associated with THGT. Among the nine significant QTLs associated with PHT in the study of Shiringani et al. (2010), one was found to be located on chromosome 2 which corresponds to the present result where locus mSbCIR223 on chromosome 2 found to be associated with PHT. Among the five QTLs detected with 50%FL in the study of Shiringani et al. (2010), two of them were located on chromosome 6 and 7. Similar results were obtained from the present study where markers Xtxp145 on chromosome 6 and Xtxp278 on chromosome 7, both significantly associated with 50% FL. Thus, some of the QTLs detected in previous reports were also detected in the present study. However, there are also some discrepancies between the findings of the present study and the previous QTL mapping studies. For example, in Srinivas et al. (2009), QTLs controlling for PWT and GWPP were detected on chromosome 6 where as in the present study PWT, GWPP and PHI were associated with locus gbsp123 on chromosome 8. Moreover, in both Srinivas et al. (2009) and Shiringani et al. (2010), several QTLs simultaneously localized on more than three chromosomes were detected for each trait whereas in the present study only PHT, 50%FL, and THGT were associated with more than one chromosome simultaneously. This discordance suggests two possible reasons; one reason is that this study may not have detected all the existing major QTLs because of the small number of markers used. Another cause is that a major QTL detected by a bi parental cross QTL mapping may not have large effect in the phenotypic variation of a germplasm collection and may be difficult to be detected with association mapping approach (Shahzad et al., 2009). Beyond the causes of discrepancy issues, the above presented cases noticed useful insight in the application of plant breeding. The significant association





**Figure 5.** Chromosomal regions of marker trait associations. The linked markers highlighted in red are the present findings where as those in black are previously identified ones. Physical positions in base pairs (Mbp) are indicated on the left of the map and the corresponding marker names are indicated on the right. The sources for marker positions are Mace et al. (2009) and Wang et al. (2012).

of *gsb123* with PWT, GWPP and PHI indicated that this locus on chromosome8 simultaneously influenced the expression of the three traits notifying the presence of pleiotropic effect (*Xtxp145* on chromosome6 showed the same pleiotropic effect on 50%FL and PHT). This implies that in variety development improving for one trait helps for the improvement of the other which simplifies fixation in breeding materials. On the other hand, the expression of 50%FL, PHT, and TGHT were controlled by QTLs at different chromosomes indicating the presence of epistatic effects. A direct implication of epistasis is that the effects of the single-locus QTLs are mostly dependent on the genotypes of other loci, and the effect of a QTL can sometimes be negated by the genotypes of a second locus; thus an attempt for utilization of these QTLs in the breeding programs has to take in to account for such epistatic effects (Xing et al., 2002). To sum up, despite some discrepancies, many of the loci that were identified in this study were associated with traits common with previous studies indicating the creditability of the results, though relatively small number of markers used. Also, though the percentages of the variance explained by the associated markers are seemingly low ranging from 2.6 to 17.76% (Table 1), these estimates are lower than the

real QTL effects because in association mapping approach incomplete LD between marker and QTL leads to an underestimation of the variance explained by the QTL (Wurschum, 2012). Comparable results between biparental mapping population QTL analysis and association mapping could be observed when LD is perfect ( $r^2=1$ ) and the same alleles segregate in both populations (Myles et al., 2009). Even if LD was perfect, underestimation of the phenotypic variance could arise from allelic frequency differential in the association mapping population (Stich et al., 2008). The maximum proportion of the variance explained by a marker is observed for allele frequencies of 0.5, as expected in biparental populations such as recombinant inbred lines or  $F_1$ -derived doubled haploids. For a germplasm collection, the allele frequencies are expected to be considerably different from 0.5, especially when multi-allelic markers such as SSRs are used (Stich et al., 2008). Thus, the proportion of the variance explained by a marker is notably lower in association mapping approach despite the same underlying allelic effect (Stich et al., 2008). Taking this into consideration, the associated markers in this study can facilitate marker assisted selection and gene introgression to develop desirable cultivars in

sorghum and the study can serve as an initial effort in Ethiopia to select and map desirable genotypes or alleles using association mapping approach. However, validation of the associated markers by increasing the marker density and evaluating the phenotypes in representative environments will improve the variance explained by the associated markers and provide a more accurate estimation of the impact that the favorable alleles will have in a breeding program. In the present study, the identified seven SSR markers (Xcup53, mSbCIR223, Xtxp114, mSbCIR248, Xtxp145, Xtxp278, and gbsp123) were localized (Voorrips, 2002) on chromosomes 1, 2, 3, 5, 6, 7 and 8, respectively, harboring one marker each (Figure 5).

## Conclusion

This study was conducted to identify drought tolerant sorghum genotypes, map chromosomal regions (QTLs) associated with agronomically important traits under moisture stress and identify SSR markers tightly linked with these QTLs. One hundred sixty (160) sorghum genotypes were evaluated in the field at Kobo in the off-season using irrigation in an alpha lattice design replicated three times. The phenotypic data were collected including days to 50% flowering, plant height, panicle weight, grain weight, grain weight per panicle, panicle harvest index, one thousand grain weight and number of grains per panicle. Analysis of variance showed highly significant ( $P < 0.0001$ ) differences among the genotypes for all characters. Most of the characters showed moderate to high phenotypic and genotypic coefficient of variation. Heritability was high for all of the studied characters. Linkage disequilibrium (LD) analysis showed that in all accessions, 107 locus pairs (32.92%) had a significant ( $p < 0.05$ ) mean LD of 0.19, with an  $R^2 > 0.2$  for 33 evaluated locus pairs. Population structure analysis showed four distinct clusters in the studied materials. A total of 10 marker-trait associations were identified using 7 different SSR markers with  $R^2$  ranging from 2.64 to 17.76. The seven SSR markers were localized on chromosomes 1, 2, 3, 5, 6, 7 and 8 harboring one marker each (xcup53, bSbCIR223, Xtxp114, mSbCIR248, Xtxp145, Xtxp278, and gbsp123, respectively).

Most of the identified markers were localized in chromosomal positions that have been previously reported as positions for drought tolerance-related traits, supporting the present findings. Hence, the associated SSR markers are potential candidates for marker assisted selection to improve drought tolerance in sorghum. Therefore, based on further validation in independent or related populations and in different environments, the markers that showed association with traits can be used to select genotypes with desirable features for a trait and land races which were found to be superior in their performance can be used for developing new varieties.

## Conflict of interests

The authors have not declared conflict of interests.

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**Supplementary Table 1.** Regions from which the accessions were collected.

Region	Afar (1)	Amhara (2)	Eritrea(3)	Gambella(4)	NA(5)	Oromia(6)	RV (7)	SNNS(8)	Tigray (9)	Total
No. of acc.	2	47	4	13	6	41	8	18	21	160

Numbers in brackets are code numbers used to represent the names of regions. RV = Released varieties, NA = Information not available, and No. of acc. = number of accessions.

**Supplementary Table 2.** Mean monthly temperature and rain fall data at the experimental site during the cropping period (January-June 2010/2011).

Month	Temperature (°C)		Rain fall (mm)	
	Minimum	Maximum	Minimum	Maximum
December	5.5	20.55	-	-
January	6.75	21.35	-	-
February	6.0	22.75	0.85	2.3
March	8.75	23.85	1.15	3.15
April	9.5	24.5	1.5	31.05
May	10.5	25.15	2.5	5.5
June	12.5	27.05	1.15	2.5
Mean	8.5	23.6	1.43	8.9

## Full Length Research Paper

# Isolation and characterization of heavy metals resistant *Rhizobium* isolates from different governorates in Egypt

Khalid S. Abdel-lateif<sup>1,2</sup><sup>1</sup>Department of Genetics, Faculty of Agriculture, Menoufia University, Egypt.<sup>2</sup>High Altitude Center, Taif University, Saudi Arabia.

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Contamination of soil with heavy metals is one of the major environmental problems in many countries that reach from many sources as power stations, application of metal pesticides, fertilizers, and sewage sludge. In this study, ten *Rhizobium leguminosarum* bv. *viciae* isolates were collected from different governorates in Egypt (Menoufia, Kafr El-Sheikh, Qalubia, Fayoum, Ismailia, Sharkya, Dakhalia, Behira and North Sinai) to evaluate their resistance to three heavy metals (Cu, Zn and Pb). The results showed that the isolates of RL3 and RL6 exhibited the best resistance toward the heavy metals tested. PCR based specific primers were used to screen the tested isolates for detection of some heavy metal resistant genes (*copA*, *pbrA* and *czcD*). The Pb-resistant gene *pbrA* was detected in most of tested isolates except RL7 and RL9 isolates, while, the Cu-resistant gene *copA* was found in all isolates except RL1, RL2, RL4 and RL8 isolates; however, the Zn-resistant gene *czcD* was detected only in the RL9 isolate. SDS-PAGE analysis was used to study the protein banding patterns for some tested isolates under lead stress and compared them with their untreated control.

**Key words:** *CopA*, *pbrA* and *czcD* genes, heavy metals, *Rhizobium*.

## INTRODUCTION

*Rhizobia* are Gram-negative soil bacteria with high agronomic significance due to their ability to establish nitrogen-fixing symbiosis with leguminous plants through invading their roots and forming nodules for atmospheric nitrogen fixation (Stan et al., 2011). The symbiosis process can be affected by many environmental factors such as temperature, soil acidity and salinity (Dart, 1977; Gibson and Jordan, 1983; Sobti et al., 2015). Heavy metals soil contamination is among the factors that have

negative effects on the growth of each rhizobium and plant. Nowadays, with increasing industrial activities, the use of industrial waste waters for irrigation and application of metal containing pesticides and fertilizers, the level of soil pollution with heavy metals is increased (Gopalakrishnan et al., 2014; Stan et al., 2011). Approximately 30% of the terrene environment is suggested to be degraded or contaminated and this surely can cause disaster problems for each environment

E-mail: k\_dein2001@yahoo.com.

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and agricultural production (Alloway and Trevors, 2013; Valentín et al., 2013). The exposure to heavy metals is toxic not only for soil microorganisms but also for plants. It has been showed that with increasing concentrations of heavy metals such as Cu, Zn and Pb, the bacterial counts of *Rhizobium sp.* are reduced and also the expression of nod genes was varied (Stan et al., 2011; Chaudri et al., 2008). On the contrast, it was suggested that *Rhizobia* can tolerate high heavy metal concentrations in different ways and may play a significant role in the restoration of contaminated soil (Carrasco et al., 2005; Teng et al., 2015). The symbiotic relationship between rhizobia and legumes reinforce elimination rate of pollutants (Glick, 2010). Hao et al. (2014) showed that rhizobia heavy metal tolerance mechanisms may include: (i) Adsorption and accumulation of heavy metals; and (ii) microbial secretion of enzymes and bioactive metabolites to increase their bioavailability and sequester their toxicity.

In this study, ten rhizobial isolates were collected from root nodules of broad bean (*Vicia faba* L.) plants representing different geographic sites in Egypt. The objectives of this study was to: (i) characterize these isolates by comparing their growth on medium supplemented with different concentrations of heavy metals (Cu, Pb and Zn); and (ii) screen the tested isolates for presence of heavy metal resistance genes using polymerase chain reaction (PCR) and to study the protein banding patterns under some heavy metal stress using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

## MATERIALS AND METHODS

### Isolation of *Rhizobia*

Ten isolates of *Rhizobium leguminosarum* bv. *Viciae* (RLV) were collected from root nodules of *V. faba* L. plants representing several Egyptian governorates according to the methods described by Vincent, 1970. Table 1 shows the isolation sites and the name of the isolates.

### DNA extraction

Total genomic DNA was extracted from bacterial cultures grown in yeast extract mannitol media (YEM) as described by Shamseldin et al. (2009). The quality and quantity of DNA was characterized both spectrophotometrically and by 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

### Amplification of heavy metals resistant genes

The primers for the amplification of a Cu-resistance gene (*pcoR*) were *pcoRf*; 5-CAGGTCGTTACCTGCAGCAG-3(forward) and *pcoRr*; 5-CTCTGATCTCCAGGACATATC-3(reverse) (Trajanovska et al., 1997). Pb-resistant gene (*pbrA*) primers were *pbrA1*; 5-ATGAGCGAATGTGGC TCGAAG-3(forward) and *pbrA2*; 5-TCATCGACGC AACAGCCTCAA-3(reverse) (Borremans et al.,

**Table 1.** Sources of *Rhizobium* isolates.

<i>Rhizobium</i> isolates	Geographical origin
RL1	Menoufia governorate
RL2	Kafr El-Sheikh governorate
RL3	Qalubia governorate
RL4	Gharbia governorate
RL5	Fayoum governorate
RL6	Ismailia governorate
RL7	Sharkya governorate
RL8	Dakhalia governorate
RL9	Behira governorate
RL10	North Sinai governorate

2001). Zn-resistant gene (*czcD*) primers were *czcD1*; 5-CAGGTC ACTGAC ACG ACC AT-3(forward) and *czcD1*; 5-CAT GCT GAT GAG ATT GAT GAT C-3(reverse) (Nies et al., 1989). PCRs were carried out in 25 µl reaction mixtures using the following conditions: Initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 3 min at 72°C. A final extension was done at 72°C for 7 min. PCR products were separated on 2% agarose gels at 100 V for 1 h in TBE buffer, stained with ethidium bromide, and photographed under UV light.

### Evaluation of heavy metals tolerance

The RLV isolates were evaluated for their tolerance against three different heavy metals (Cu, Zn and Pb) by plating in YEMA medium. The stock solutions of heavy metals (mM) were added to sterile agar as follows: CuCl<sub>2</sub>·2H<sub>2</sub>O 0.5, 1 and 2; ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.5, 1 and 2; Pb(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·3H<sub>2</sub>O 0.5, 1.0 and 2. The plates were inoculated with bacterial cells and the bacterial growth was evaluated after 7 days at 28°C (Ausili et al., 2002). Isolates were considered resistant if growth was observed or sensitive if otherwise.

### Protein banding patterns of Rhizobial isolates

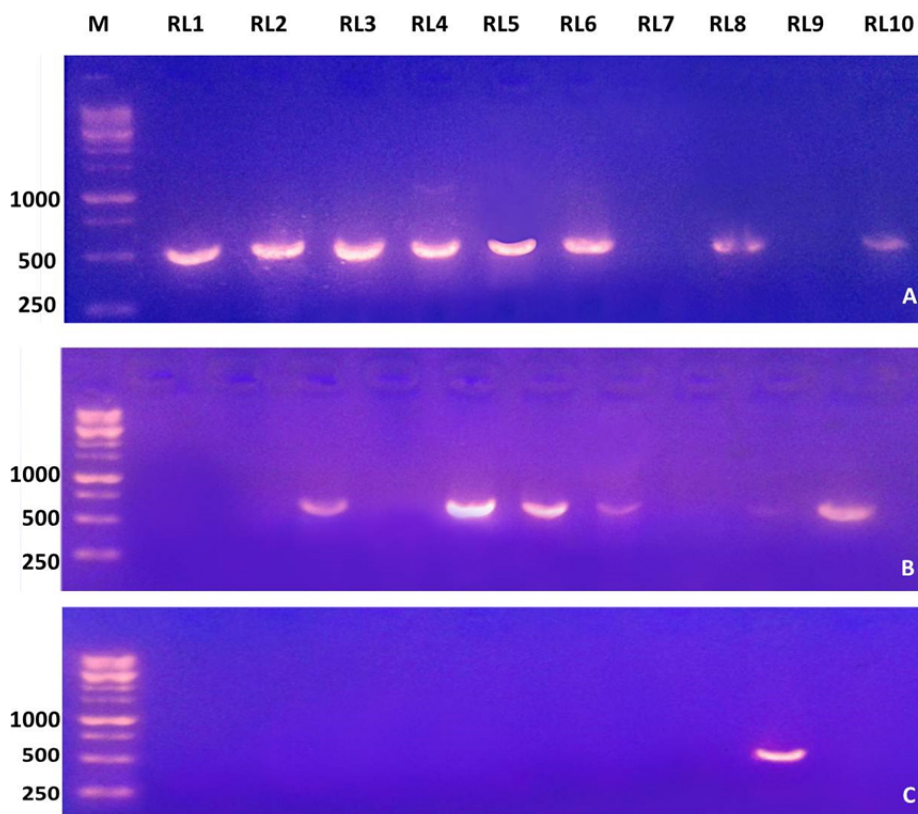
The cultures of tested isolates growing on Broth YEM medium and supplemented with 0.5 mM of Pb(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·3H<sub>2</sub>O were pelleted and Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Pereira et al. (2006). Gels were stained with Coomassie brilliant blue R-250 and photographed.

## RESULTS AND DISCUSSION

### Detection of some heavy metal resistant genes

In this study, PCR based on heavy metal specific primers was used to screen and detect some heavy metal resistant genes in the tested isolates (*copA*, *pbrA* and *czcD*). The primers used for the amplification Pb-resistant gene *pbrA* yielded a band of approximately 500 bp in most of tested isolates except RL7 and RL9 (Figure 1A). For Cu-resistant gene *copA*, the PCR produced one band of approximately 650 bp in most of tested isolates except RL1, RL2, RL4 and RL8 (Figure 1B). This band is homologous to that obtained by Trajanovska et al. (1997).





**Figure 1.** Amplification of heavy metal resistant genes. A, Amplification of the Pb-resistant gene, *pbrA*; B, amplification of Cu-resistant gene *copA*; and C, amplification of Zn -resistant gene *czcD*.

On the other side, only one band of approximately 500 bp was detected in RL9 isolate with amplification of the Zn -resistant gene *czcD* (Figure 1C). It must be mentioned that microbes use heavy metal resistant genes to encode products that reduce or eliminate the toxicity of heavy metals for adaptation of the different environment stresses (Wei et al., 2009). From these genes, *pbrA*, which encodes a P-type Pb(II) efflux ATPase in the lead resistance operon that involved in uptake, efflux, and accumulation of Pb(II) (Borremans et al., 2001). In addition, CopA is essential gene in copper resistance operon and catalyzes the intake of copper (Wei et al., 2009). Moreover, the *CzcDis* necessary gene in Zn-resistant operon and mutation in this gene can disrupt level of Zn-resistance (Nies et al., 1989).

#### Screening of *Rhizobium* isolates for heavy metals resistance

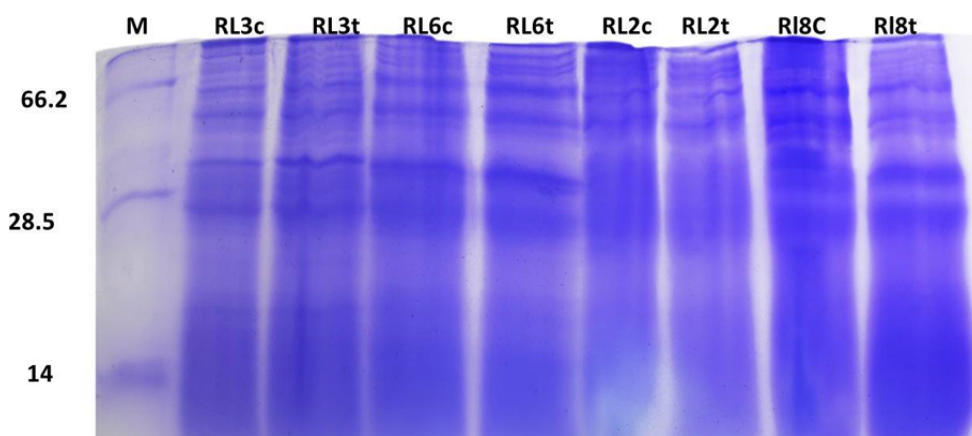
All of *Rhizobium* isolates were tested for their resistance to heavy metals using concentrations of 0.5, 1 and 2 mM of Cu, Pb and Zn (Table 2). The isolates were considered to be resistant when the growth occurs in the presence of heavy metals or sensitive if otherwise. First with Pb

treatment, all isolates were able to grow at the low concentration (0.5 mM), while most of the isolates were found to be resistant to Pb at 1 mM except RL2, RL8 and RL9 isolates. At the highest concentration of lead (2 mM), most of isolates failed to grow except RL3 and RL6 isolates. For Cu treatment, all isolates were able to grow at the low concentration (0.5 mM) except RL8 and RL10 isolates. Moreover, only the isolates RL1, RL4 and RL6 were succeeded to grow at concentration of 1 mM of Cu, while no isolates appeared at the highest concentration of Cu (2 mM). Finally, for Zn treatment, all of isolates succeeded to grow at 0.5 mM except the isolate RL7, while most of isolates can grow at 1 mM of Zn except RL7, RL8 and RL10 isolates. Furthermore, no isolates appeared at the highest concentration of Zn (2 mM) in general, the isolates RL6 and RL 3 showed the highest levels of Pb resistance, while RL1, RL 4 and RL6 isolates exhibited the highest levels of Cu resistance. In addition, RL3, RL 5, RL6 and RL9 were the best isolates in Zn resistance. It must be mentioned that the ability to resist the heavy metals decreased with increasing their concentrations. These results are consistent with previous studies shown that the increased concentrations of heavy metals can affect the growth, morphology and activities of microorganisms in nitrogen fixation (Khan and Scullion,

**Table 2.** Effects of different concentrations of heavy metals on the growth of *Rhizobial* isolates on YEMA plates.

Isolates	Control	Pb (mM)			Cu(mM)			Zn(mM)		
		0.5	1	2	0.5	1	2	0.5	1	2
RL1	++++	++	+	-	+++	+	-	++	+	-
RL2	++++	++	-	-	+++	-	-	++	+	-
RL3	++++	++	+	+	+++	-	-	++	++	-
RL4	++++	++	++	-	++	+	-	+	+	-
RL5	++++	+++	++	-	+	-	-	++	++	-
RL6	++++	++	++	+	+	+	-	++	++	-
RL7	++++	+++	+	-	+	-	-	-	-	-
RL8	++++	++	-	-	-	-	-	+	-	-
RL9	++++	++	-	-	+++	-	-	++	++	-
RL10	++++	++	+	-	-	-	-	+	-	-

Growth: +++++, very good; +++, good; ++, moderate; +, poor; -, no growth.



**Figure 2.** SDS-PAGE protein banding patterns of some *Rhizobium* isolates grown in broth YEM medium without treatment (c) and treated with 0.5 mM Pb (t). M, protein molecular weight marker (Jena Bioscience GmbH, Germany).

2002; Lakzian et al., 2002; Shi et al., 2002; Pereira et al., 2006). Furthermore, the tested isolates exhibited more sensitive against Cu than the other heavy metals Pb and Zn. These observations are in agreement with previous studies showed that the high concentrations of Cu are toxic to soil microorganisms by affecting their structural diversity and metal tolerance (Cervantes and Gutierrez-Corona, 1994; Dell'Amico et al., 2008).

The appearance of resistance levels in isolates as RL 3 and R6 against some heavy metals as Pb is due to these isolates contain the pb-resistant gene *pbrA* (Figure 1A). On the other hand, the appearance of resistance levels in some isolates although their resistance genes did not detected by PCR is not understood. One of explanations is that these isolates were isolated from soil polluted with heavy metals and have probably adapted this environmental stress. This explanation is supported with previous literature shown that the selective pressure of metals on microorganisms can lead to microbial populations with a high resistance to metals (Pereira et

al., 2006). Another explanation, these isolates may have other resistance mechanisms for removal of these elements (Wei et al., 2009; Teng et al., 2015).

### Characterization of *Rhizobium* isolates by SDS-PAGE

The analysis of protein alterations seems to be a good indicator to estimate the level of stress imposed to *Rhizobium* populations (Pereira et al., 2006). Hence, SDS-PAGE analysis was used to study the protein banding patterns for some *Rhizobium* isolates under lead stress and compared them with their untreated control. The isolates RL2 and RL8 were selected as sensitive isolates, while the isolates RL3 and RL 6 were selected as resistant according to their growth under lead stress (Figure 2). In general, *Rhizobium* isolates showed similar banding patterns however some differences were detected as indicated in Figure 2. Comparative analysis of the lanes showed the absence of two protein bands



(about 25 and 40 kDa) in the sensitive isolate RL2 (treated and their control) compared to other isolates.

## Conclusion

Isolation of rhizobia strains resistant to stresses like heavy metals is very important for efficient nitrogen fixation and improving plant productivity especially in the contaminated areas. The results of this study showed in general that the isolates of RL3 and RL6 found to be the best isolates to tolerate Pb, Cu and Zn heavy metal elements. Future studies must be done to test these isolates in fields contaminated with heavy metals for increasing nitrogen fixation level by *faba* bean plants cultivated in contaminated soils.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Morphometric characterization of *Jatropha curcas* germplasm of North-East India

Adreeja Basu<sup>1\*</sup>, Lokanadha Rao Gunupuru<sup>2</sup> and Lingaraj Sahoo<sup>1,3</sup>

<sup>1</sup>Center for Energy, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India.

<sup>2</sup>Molecular Plant-Microbe Interactions Laboratory, School of Biology and Environmental Science, University College Dublin, Dublin 4, Ireland.

<sup>3</sup>Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India.

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The morphological variation among *Jatropha curcas* L. populations from 29 different locations of North-East India was determined. Four populations from other parts of India were also incorporated in the study as an out-group. The morphological trait based analysis of *J. curcas* revealed large variation of quantitative traits among the populations. Of the six morphological traits used in this study, the highest variation (% coefficient of variation=23.19) was observed in floral sex ratio (M: F) while canopy spread (CS) appeared as the least variable trait (% coefficient of variation=1.67). Based on morphometric trait values, the three populations from Assam (IITJC15, IITJC24 and IITJC28) and one population from Arunachal Pradesh (IITJC7) emerged as superior when compared with the other populations. Both cluster and principal component analyses depicted that the populations IITJC19, IITJC21, IITJC22 and IITJC24 from Assam maintained maximum inter-cluster distance from the rest of the populations and are, thus, substantially distinct. The analyses also depicted that no clear demarcation can be made between populations from North-Eastern India and other areas on the basis of morphometric variability alone. Morphometric characterization of *J. curcas* populations leads to the identification of seven promising populations (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) from North-East India which can substantially contribute to *Jatropha* breeding in the future. From the study of variance components and broad sense heritability, it was suggested that the selection of elite plants on the basis of M: F ratio, 100 seed weight (100SW) and total seed yield (TSY) in tree improvement programs is likely to be more successful.

**Key words:** Agronomic traits, cluster analysis, principal component analysis, heritability, ANOVA.

## INTRODUCTION

*Jatropha curcas* (Euphorbiaceae) has recently gained worldwide importance as a sustainable source of

biodiesel. The fuel obtained from *J. curcas* seed oil is an important replacement of petroleum-based diesel fuel

\*Corresponding author. E-mail: adreejabasu.iitguwahati@gmail.com. Tel: +91-9748882018.

(Chitra et al., 2005). The fact that *J. curcas* seed oil upon transesterification can provide better quality biodiesel with high cetane number when compared with other oil-seed plants has created a surge of interest in this plant. *J. curcas* plant is a native of Mesoamerica, however, it has been distributed throughout the arid, semi-arid, tropical and subtropical regions of the world (Augustus et al., 2002). *J. curcas* was introduced to India by Portuguese seafarers during the sixteenth century (Sunil et al., 2008). In India, *J. curcas* has acclimatized itself in diverse eco-geographical zones with different edapho-climatic conditions and, consequently, has over time amassed variability within the germplasm (Bhatt et al., 2014).

Evaluation of morphological traits of *J. curcas* provide ample information of the genetic diversity of *J. curcas* population (Divakara et al., 2010; Kaushik et al., 2007; Rao et al., 2008). Studies focussing on agronomic traits, qualitative traits, reproductive traits and genetic variability are of utmost significance for a thorough investigation of extent of phenetic diversity in germplasm and for utilizing the genetic information for crossing and breeding programmes (Franco et al., 2001; Montes and Melchinger, 2016). For successful crop enhancement and commercial exploitation of the biofuel plant, *J. curcas* improvement programs should aim at agronomic traits like low male to female flower ratio, high seed yield and oil content, abiotic and biotic stress resistance, and high natural ramification of branches with greater canopy spread. *J. curcas* crop improvement programs are largely dependent on the assessment of variability in wild sources and selection of superior genotypes (Divakara et al., 2010). Presently, *J. curcas* varieties with elite traits which can be grown in varied conditions in different parts of the world are not available for the growers, which makes this crop a risky business (Jongschaap et al., 2007; Moniruzzaman et al., 2016). Thus, assessment of trait based variability in *J. curcas* is a prerequisite for the screening and selection of agronomically elite genotypes which can later be exploited in breeding programs. To date, phenotypic diversity studies in *J. curcas* have mainly focused on the assessment of variability in seed traits and seed-oil content (Ginwal et al., 2005; Kaushik et al., 2007; Mazumdar et al., 2012). However, systematic studies involving other morphological and agronomically important traits in *J. curcas* germplasm such as plant height, canopy spread, male to female flower ratio, collar length, total seed yield and 100 seed weight for selection of elite planting material have been rare (Rao et al., 2008; Srivastava et al., 2011).

The North-East region of India comprises eight states viz., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. Diverse climatic conditions (tropical to temperate), high variation in altitude (50 m to 7000 m) along with high rainfall (800 mm to 4000 mm) has made this agroclimatic zone one of the richest reservoirs of plant diversity in the country. In

North-East India, *J. curcas* grows mainly in the wild and such plant populations, with a longer history of isolation and evolution, harbor a significant amount of diversity (Ranade et al., 2008; Goswami and Choudhury, 2015). Such diverse populations can contribute towards broadening of *J. curcas* genetic resources and so, morphometric trait-based characterization of *J. curcas* from distinct eco-geographical regions of North-East India has the potential of identifying agronomically elite *J. curcas* plants. Unfortunately, scant research has been conducted on the morphological characterization of *J. curcas* germplasm from North-East India (Saikia et al., 2009). The main objective of this study was to assess the morphometric diversity of *J. curcas* germplasm from North-East India along with the identification of elite populations.

## MATERIALS AND METHODS

### Collection of plant material

Field trips were undertaken during the months of June-August, 2012 to gather *J. curcas* seeds. The latter were collected from six states of North-East India (viz., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram and Tripura) covering 29 distinct eco-geographical regions. *J. curcas* seeds from four other states of India viz., Delhi, Punjab, Gujarat and Orissa, were also included in the study as an outgroup (Table 1). The experiments were carried out at Centre for Energy, IIT Guwahati with the experimental site co-ordinates (26°11'5" N; 91°40'9" E) which experiences a warm temperate climate with an average annual rainfall of 1698 mm. The region comes under the influence of southwest monsoon from first week of June to early September.

The collected seeds were planted in polythene bags containing mixture of sand, soil and vermiculite in the ratio of 1:1:1 (by volume) for germination. The polythene bags were labelled according to the region of seed collection. After two months, the hardened and rooted plantlets were transferred into garden soil (pit size 50 x 50 x 50 cm) in the field of Centre for Energy, IIT Guwahati, in a randomized complete block design. The spacing between the plants was 2.5 m x 2.5 m. Ten different plants from each region were treated as a single population and assigned an accession number (Table 1). The type of soil in the field plots were a mixture of sandy laterite clay soil and very deep well drained forest clay soil. Irrigation was performed initially for two weeks manually followed by natural rain water irrigation from the monsoon rains.

### Measurement of plant morphometric characters

The study on the phenotypic assessment of morphological descriptors plant height (PH), collar diameter (CD), canopy spread (CS) and floral sex ratios (M:F) were conducted during the flowering phase of growth year three for 33 *J. curcas* populations with ten plants per population. PH, CD and CS were measured using standard measuring tape. CD was calculated from the girth measurement of the main stem 5 cm above ground. The M:F ratio was calculated counting the male and female flowers in the inflorescences. The mature seeds were harvested from the same plants and sun-dried to constant weight. The weight of 100 seeds (100SW) and total seed yield (TSY) from ten plants per population were estimated. The seeds were separated from the fruit mechanically and cleaned manually to remove all foreign material. The cleaned seeds were dried under similar temperature (35°C)

**Table 1.** Geographical locations of *Jatropha curcas* used in diversity analysis.

S/N	Population number	Location of collection	Geographical Location	
			Latitude (°N)	Longitude (°E)
1	IITJC1	Delhi	28-46	77-23
2	IITJC2	Ahmedabad (Gujarat)	23-03	72-58
3	IITJC3	Patiala (Punjab)	30-33	76-40
4	IITJC4	Bhubaneswar (Orissa)	20-15	85-50
5	IITJC5	Pasighat (East Siang, Arunachal Pradesh)	28-07	95-33
6	IITJC6	Itanagar (Papum Pare, Arunachal Pradesh)	27-08	93-40
7	IITJC7	Naharlagoon (Papum Pare, Arunachal Pradesh)	27-10	93-70
8	IITJC8	Hiangtam (Churachandpur, Manipur)	24-05	93-57
9	IITJC9	Imphal (Imphal West, Manipur)	24-48	93-56
10	IITJC10	Wangoi (Imphal West, Manipur)	24-65	93-89
11	IITJC11	Mamit (Mamit, Mizoram)	23-56	92-29
12	IITJC12	Mawhati (RiBhoi, Meghalaya)	25-49	92-50
13	IITJC13	Tura (West Garo Hills, Meghalaya)	25-30	90-16
14	IITJC14	Agartala (West Tripura, Tripura)	23-50	91-23
15	IITJC15	Amingaon (Kamrup, Assam)	26-11	91-40
16	IITJC16	Sitara (Rangia, Assam)	26-44	91-61
17	IITJC17	Sondora (Kamrup, Assam)	26-08	99-56
18	IITJC18	Samota (Nalbari, Assam)	26-44	91-44
19	IITJC19	Tezpur (Sonitpur, Assam)	26-40	92-45
20	IITJC20	Kharigaon (Kokrajhar, Assam)	26-41	90-27
21	IITJC21	Teok (Jorhat, Assam)	26-80	94-39
22	IITJC22	Makum (Tinsukia, Assam)	27-50	95-45
23	IITJC23	Mathurapur (Sibsagar, Assam)	26-98	94-88
24	IITJC24	Numaligarh (Golaghat, Assam)	26-63	93-75
25	IITJC25	Dhubri (Dhubri, Assam)	26-02	89-59
26	IITJC26	Bihpuria (Lakhimpur, Assam)	27-03	93-90
27	IITJC27	Kuruwa (Darrang, Assam)	26-13	91-46
28	IITJC28	Bokajan (Karbi Anglong, Assam)	26-01	93-78
29	IITJC29	Silchar (Cachar, Assam)	24-49	92-48
30	IITJC30	Raha (Nogaon, Assam)	26-23	92-51
31	IITJC31	Jagi Road (Marigaon, Assam)	26-61	92-12
32	IITJC32	Salmala (Bongaigaon, Assam)	26-35	90-63
33	IITJC33	Sualkuchi (Kamrup, Assam)	26-16	91-57

and humidity conditions to reach constant weight. All the quantitative data was statistically analyzed and the mean value of each morphological trait for individual populations, standard error and coefficient of variation was calculated (Table 2).

### Statistical analysis

Phenotypic inter-relations using the quantitative data were assessed using Manhattan dissimilarity coefficients. The latter were calculated as,  $M_{ij} = 1/n \sum_{k=1}^n |X_{ki} - X_{kj}|$ , where  $X_{ki}$  and  $X_{kj}$  are the observed values of two populations  $i$  and  $j$ , with respect to the  $k$ th trait, and  $n$  is the number of morphometric traits considered. The pairwise dissimilarity matrix based on the Manhattan coefficient was subjected to cluster analysis using unweighted pair group method with arithmetic mean analysis (UPGMA) (Sneath and Sokal, 1973). Principal Components Analysis (PCA) was performed to further

elucidate phenotypic variability of *J. curcas*. All calculations were performed using NTSYS-pc version 2.02 (USA) (Rohlf and Version, 1997). Pairwise Pearson correlation coefficients were computed using SigmaPlot 11.0 for the determination of the linear relationship among the morphometric traits (Wass, 2009). The analysis of variance (ANOVA), broad-sense heritability, phenotypic and genetic variance was calculated for the six selected quantitative traits using the online software PBSTAT 1.2 (Syukur et al., 2015).

## RESULTS AND DISCUSSION

### Determination of mean, minimum and maximum values and coefficient of variations

The examination of all six quantitative traits exhibited

**Table 2.** Morphological characterization of *Jatropha curcas* populations.

S/N	Sample code	Mean PH (cm)	Mean CD (cm)	Mean CS (cm)	Mean M:F ratio	Mean 100SW (g)	Mean TSY (g)
1	IITJC1	185.4±0.95	18.6±0.45	120.4±0.63	24.0:1	54.4±0.81	110.8±0.72
2	IITJC2	168.4±1.94	11.4±0.40	116.1±0.54	16.5:1	68.4±0.96	149.9±1.38
3	IITJC3	180.8±1.10	14.8±0.35	138.6±1.04	22.1:1	72.7±1.00	98.4±0.95
4	IITJC4	143.0±0.94	12.8±0.29	104.5±0.61	16.0:1	76.5±1.03	158.8±1.07
5	IITJC5	162.4±1.37	14.5±0.35	129.6±0.89	14.3:1	78.8±0.94	180.2±1.11
6	IITJC6	280.6±2.24	24.0±0.69	137.4±0.68	13.2:1	96.8±0.91	196.8±1.18
7	IITJC7	268.7±1.90	23.7±0.67	148.4±0.88	14.1:1	123.8±1.01	166.7±1.19
8	IITJC8	269.6±1.45	16.8±0.37	139.7±0.58	13.9:1	88.5±0.84	175.8±1.12
9	IITJC9	240.9±1.43	17.9±0.32	152.2±0.57	14.4:1	116.2±0.96	184.0±1.12
10	IITJC10	277.7±2.16	24.2±0.55	159.7±0.61	12.7:1	95.3±0.47	226.9±1.37
11	IITJC11	263.9±2.72	14.8±0.74	138.2±0.77	13.2:1	62.5±0.84	215.7±1.30
12	IITJC12	194.4±1.54	15.6±0.15	143.3±0.65	13.8:1	67.8±0.51	232.4±1.13
13	IITJC13	178.6±1.65	14.2±0.43	149.2±0.61	14.4:1	71.5±0.63	221.9±1.14
14	IITJC14	177.6±2.01	16.8±0.45	136.7±0.53	14.0:1	68.5±0.32	206.2±1.19
15	IITJC15	191.1±1.54	9.6±0.41	124.7±0.56	10.5:1	85.8±0.64	283.2±1.64
16	IITJC16	298.0±1.30	10.4±0.86	192.5±1.38	13.0:1	83.5±0.61	218.2±1.59
17	IITJC17	153.7±1.50	5.8±0.35	107.3±0.52	19.1:1	89.5±0.47	91.1±1.23
18	IITJC18	198.3±0.87	18.9±0.52	156.2±0.74	13.0:1	66.8±0.75	222.8±1.22
19	IITJC19	336.2±1.73	14.4±0.96	229.6±1.46	11.8:1	71.8±0.78	248.2±1.08
20	IITJC20	223.7±1.67	13.6±0.33	177.8±0.54	13.3:1	69.3±0.56	225.1±1.05
21	IITJC21	329.1±2.37	16.8±0.45	237.2±1.62	13.8:1	70.5±0.57	209.1±1.21
22	IITJC22	376.4±2.16	11.8±0.41	191.9±0.55	12.9:1	74.9±0.64	231.3±1.76
23	IITJC23	287.2±1.43	8.6±0.24	149.2±0.61	25.4:1	74.7±0.51	118.8±0.81
24	IITJC24	385.7±2.03	11.8±0.59	239.9±1.47	12.5:1	71.0±0.33	216.5±1.49
25	IITJC25	282.7±1.48	15.8±0.41	147.2±0.39	16.4:1	87.5±0.59	169.2±1.32
26	IITJC26	159.8±1.53	11.4±0.89	134.8±0.48	14.0:1	78.4±0.46	207.8±1.28
27	IITJC27	310.8±1.82	8.8±0.39	145.3±0.56	12.4:1	84.4±0.71	223.9±1.99
28	IITJC28	192.3±1.19	30.5±0.95	154.8±0.47	13.7:1	108.4±1.05	200.4±1.30
29	IITJC29	182.8±0.99	15.4±0.47	141.5±0.82	16.0:1	65.3±0.47	155.6±1.00
30	IITJC30	289.8±1.28	9.5±0.49	163.4±0.90	12.5:1	79.5±0.92	235.9±1.92
31	IITJC31	258.7±1.53	7.2±0.28	132.5±0.65	11.5:1	81.1±0.69	241.6±1.60
32	IITJC32	145.9±1.50	14.6±0.38	133.2±1.24	14.1:1	74.3±0.36	198.3±1.08
33	IITJC33	255.1±1.61	9.8±0.62	149.8±0.61	19.5:1	72.5±0.92	172.2±1.73
	Mean	237.9	14.7	152.2	14.9	79.7	193.7
	CV (%)	2.13	11.07	1.67	23.19	2.75	2.05

considerable morphological variability in 33 *J. curcas* populations under investigation ( $p < 0.01$ ) (Table 2). The mean data on morphometric parameters showed broad variation in plant height (PH) (143 cm - 385.7 cm). Significant differences were observed in collar diameter (CD) (8.61 cm - 30.46 cm), seed weight per 100 seeds (100SW) (54.4 g-123.8 g) and total seed yield (TSY) (91.1 g - 283.8 g). Canopy spread (CS) appeared to be the least variable trait (104.5 cm - 239.9cm) with % coefficient of variation (%CV) of 1.67. The highest variation (%CV=23.19) was observed in male to female flower (M:F) ratio (10.5 - 25.4). The population, IITJC24, had the highest PH (385.7 cm) and CS (239.9 cm) with respect to all other populations. In contrast, IITJC15 had

a maximum TSY (283.2 g) and a minimum M: F ratio (10.5:1). IITJC28 had a maximum CD (30.5 cm). The population IITJC7 from Arunachal Pradesh recorded the highest 100SW (123.8 g). Consequently, three populations from Assam (IITJC15, IITJC24 and IITJC28) and one population from Arunachal Pradesh (IITJC7) emerged superior on the basis of morphometric trait values.

#### Correlation coefficients

The Pearson correlation between growth attributes and seed characteristics of *J. curcas* was estimated. The

**Table 3.** Pearson correlation coefficients for morphometric traits of *J. curcas* populations.

	CD	CS	M:F ratio	100SW	TSY
PH	-0.05	0.77**	-0.26	0.12	0.35*
CD		0.05	-0.12	0.42**	0.01
CS			-0.33	-0.08	0.42**
M:F ratio				-0.24	-0.87**
100SW					0.01

Significance level: \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ .

correlation coefficients revealed a positive relationship between growth traits PH, CS and CD with TSY (Table 3). However, the M: F ratio was negatively correlated with all other morphological traits. Interestingly, the existence of a highly significant negative association was observed between M:F ratio and TSY. This allows us to hypothesize that a direct positive correlation exists between the number of female flowers and TSY. This is in accordance with Rao et al. (2008) who found a positive relationship between plant height and female to male flower ratio with seed yield. The fact that IITJC3 and IITJC17, the low seed-yielding populations, scored below average value for most of the other morphological traits was in accordance with the correlation analysis data which showed a positive association between all traits (with the exception of M:F ratio) with TSY. Thus, correlation analysis allowed direct assessment of positive and negative contribution of other quantitative traits on TSY.

*J. curcas* is having a gestation lag of 3-4 years (Biswas et al., 2010). Thus, during tree improvement programs, seed-related traits cannot be used for preliminary screening of *J. curcas* planting materials from large-scale plantations at early stage of growth. Since growth related traits of *J. curcas* is having a positive correlation with TSY, it was suggested that morphometric traits like PH, CS and CD can be used as initial screening indices for the selection of *J. curcas* plants at an early stage of growth, that is, before completion of the gestation period. It was also suggested that during *J. curcas* improvement programs, increasing the total number of female flowers or producing a more extensive canopy will provide better opportunities for increasing total seed yield of the plant.

### Cluster and principal component analyses

The UPGMA dendrogram, based on the Manhattan dissimilarity matrix, separated the 33 populations into three major clusters I, II and III with 16, 13 and 14 populations respectively. Cluster I and Cluster II again formed four (IA, IB, IC and ID) and three (IIA, IIB and IIC) distinct sub-clusters respectively (Figure 1). The grouping of 33 *J. curcas* populations in eight sub-clusters is shown

in Table 4. The remaining populations dispersed themselves into sub-clusters IA and IB along with populations from Assam and Arunachal Pradesh. The sub-clusters IC, IIA and IIB consisted of mixed populations from the North-East. Conversely, sub-clusters ID, IIC and cluster III were specific to populations from Assam.

The sub-cluster wise mean values of the quantitative morphological traits were also estimated (Table 5). The highest mean plant value (356.86 cm) and mean canopy spread (224.7 cm) were observed in cluster III. The latter also showed high values for 100SW and low M: F ratio. Sub-cluster IC comprising of populations from Meghalaya, Tripura and Assam recorded the highest CD (18.3 cm). Sub-cluster IIA, consisting of two populations each from Arunachal Pradesh, Manipur and Assam, recorded the maximum 100SW (97.6 g). The highest value of TSY and the lowest M: F ratio was observed in sub-cluster ID containing a single population, IITJC15. Thus, cluster III and sub-cluster ID recorded high mean values for the majority of agronomic traits. The populations from cluster III (IITJC19, IITJC21, IITJC22 and IITJC24) and sub-cluster ID (IITJC15) were found to be promising for future tree improvement programs.

In order to gain a better understanding of the relationship between *J. curcas* populations, Principal Component Analysis (PCA) was undertaken by concurrently assessing all six morphometric traits (Figure 2). PCA showed separation of the populations into three discrete groups (I, II and III). Groups I and II were again sub-divided into four and three sub-groups respectively. The overall grouping pattern of the populations in PCA was in accordance with the major clades of the UPGMA dendrogram. It has been previously reported that the crossing of populations from the clusters, which exhibit maximum inter-cluster distance and high mean value of agronomic traits, would result in production of more divergent trees (Kaushik et al., 2007; Shabanimofrad et al., 2013; Srivastava et al., 2011). Both the analyses delineated that the four populations in cluster III, IITJC19, IITJC21, IITJC22 and IITJC24, have maintained maximum inter-cluster distance from other *J. curcas* populations. Thus, it can be theorized that the selection of parents from these four populations during breeding programs would lead to the development of *J. curcas* plants with greater genetic heterogeneity.

When the grouping pattern of outside North-East and North-East populations were compared, it was observed that in both cluster and principal component analyses, *J. curcas* populations from outside North-East have nested together with populations from North-East India. Thus, from morphological-character-derived cluster analysis and principal component analysis it was determined that for all morphometric traits, association among populations was independent of their geographic origin. The inability of the dendrogram and PCA plot to reveal a clear relationship between diversity pattern and

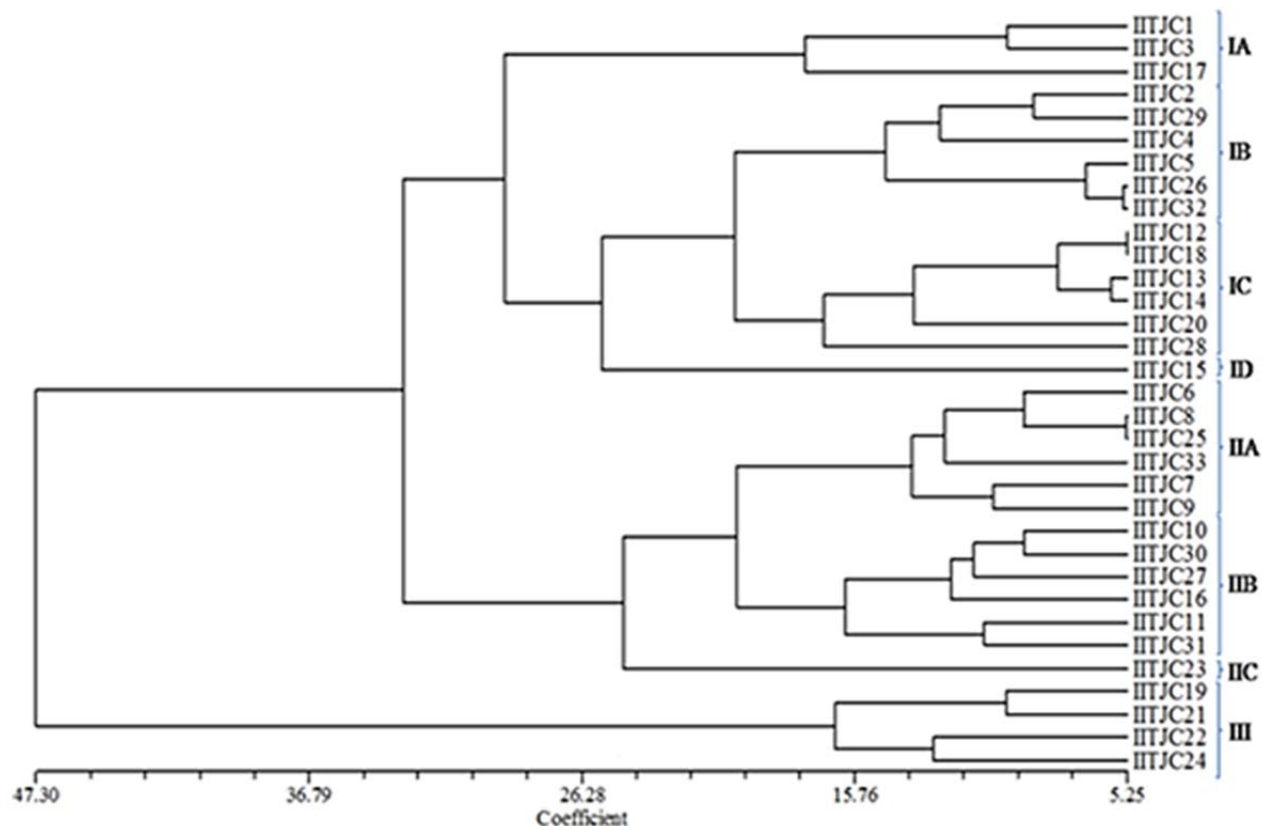


Figure 1. Morphometric relationship among 33 *J. curcas* populations based on UPGMA based cluster analysis.

Table 4. Grouping of *Jatropha curcas* populations as depicted by cluster analysis.

Clusters	Sub-Clusters	No. of populations	Population codes
I	IA	3	IITJC1, IITJC3, IITJC17
	IB	6	IITJC2, IITJC4, IITJC5, IITJC26, IITJC29, IITJC32
	IC	6	IITJC12, IITJC13, IITJC14, IITJC18, IITJC20, IITJC28
	ID	1	IITJC15
II	IIA	6	IITJC6, IITJC7, IITJC8, IITJC9, IITJC25, IITJC33
	IIB	6	IITJC10, IITJC11, IITJC16, IITJC27, IITJC30, IITJC31
	IIC	1	IITJC23
III	III	4	IITJC19, IITJC21, IITJC22, IITJC24

geographical origin led to the deduction that morphometric traits of *J. curcas* are relatively uncorrelated with geographic distribution.

**Analysis of variance and estimation of variance components**

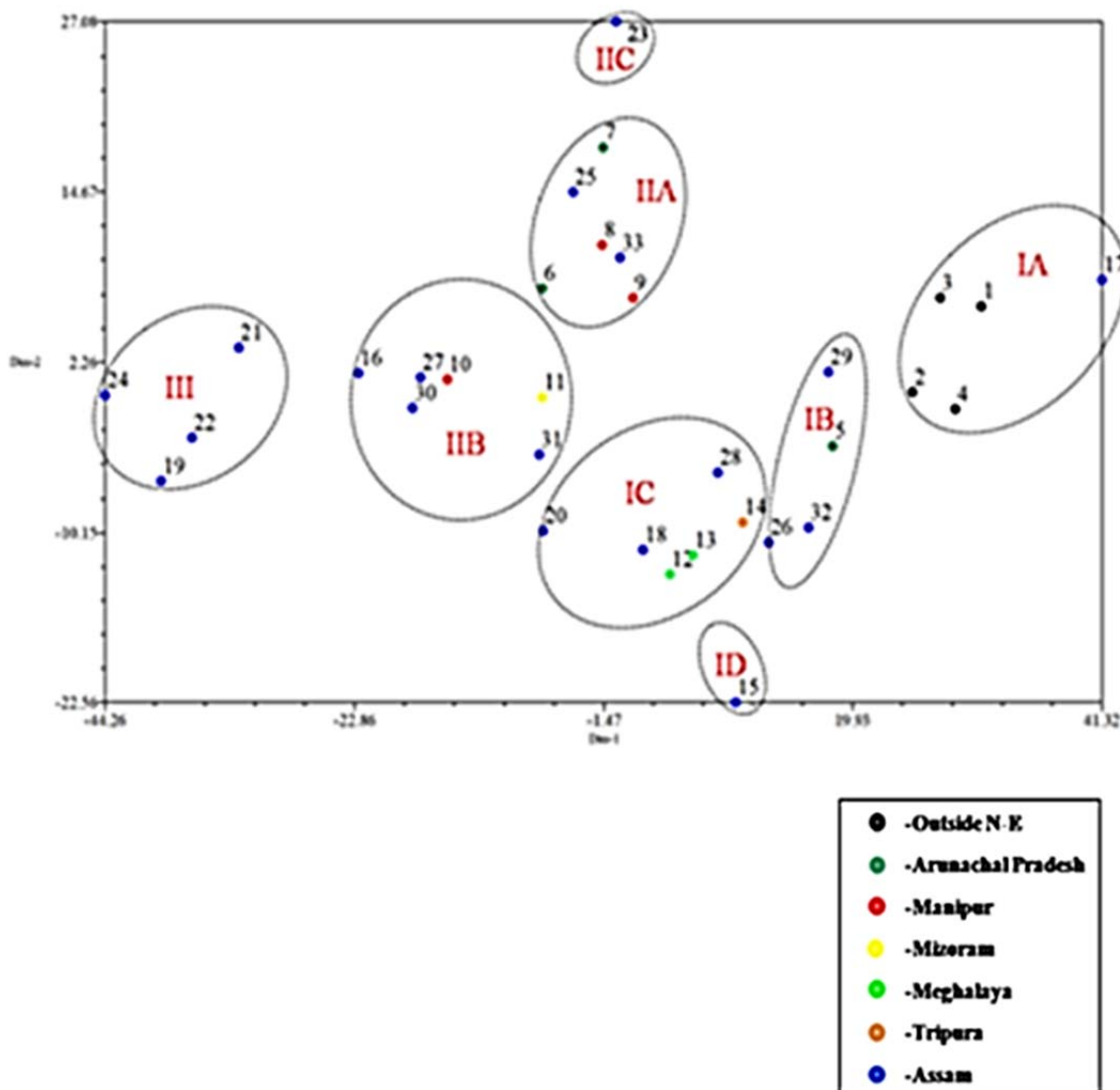
An ANOVA for morphometric traits reflected highly

significant differences between the *J. curcas* accessions under investigation at  $p \leq 0.01$  (Table 6). ANOVA among *J. curcas* accessions for different morphometric characters have previously been reported (Shabanimofrad et al., 2013; Sunil et al., 2012).

In selection and breeding experiments, knowledge of heritability and phenotypic trait under selection is essential for predicting the selection response and improving the agronomic trait (Robinson et al., 1949;

**Table 5.** Mean values of morphological traits in *Jatropha curcas* for eight sub-clusters.

Clusters	PH (cm)	CD (cm)	CS (cm)	M:F ratio	100SW (g)	TSY (g)
IA	173.3	13.1	122.1	21.7:1	72.2	100.1
IB	160.4	13.4	126.6	15.2:1	73.6	175.1
IC	194.2	18.3	153	13.7:1	75.4	218.1
ID	191.1	9.6	124.7	10.5:1	85.8	283.2
IIA	266.3	18	145.8	15.3:1	97.6	177.5
IIB	283.2	12.5	155.3	12.6:1	81.1	227
IIC	287.2	8.6	149.2	25.4:1	74.7	118.8
III	356.86	13.7	224.7	12.8:1	72.1	226.3



**Figure 2.** Two dimensional scaling of 33 *J. curcas* populations based on principle component analysis to elucidate morphometric relationship of *J. curcas*. The numbers represent the codes given for each accession of *J. curcas*.

Visser et al., 2008). Thus, broad sense heritability ( $h^2_{bs}$ ) and variance components (phenotypic variance,  $V_P$  and

genotypic variance,  $V_G$ ) were estimated for all six agronomic traits in *J. curcas* (Table 7). The estimates of



**Table 6.** Analysis of variance for morphometric traits in 33 *J. curcas* populations.

Source	Df	Mean Sum of Squares					
		PH	CD	CS	M:F ratio	100SW	TSY
Replicates	9	89.6**	8.3**	19.3**	100.2**	26.9**	63.3**
Populations	32	46001.2**	283.9**	11,000.60**	119.4**	2261.6**	19982.4**
Error	288	25.6	2.6	6.5	12	4.8	15.8

Significance level: \*\* =  $p < 0.01$ .

**Table 7.** Estimation of variance components and broad sense heritability.

Variable	$V_G$	$V_P$	$h^2_{bs}(\%)$
PH	1.9397	2.7166	71.40
CD	0.1716	0.2517	68.2
CS	0.3882	0.5847	66.39
M:F ratio	2.6735	3.0356	88.07
100SW	0.6703	0.8155	82.19
TSY	1.4394	1.919	75.01

$V_P$  as compared to  $V_G$  were higher for all traits. Broad-sense heritability is defined as the ratio of total genetic variation to total phenotypic variation (Brown et al., 2012). Thus, the high magnitude of  $h^2_{bs}$  (65%) for all morphological traits depicted the dominance of heritable variation in *J. curcas*. Comparatively high % of  $h^2_{bs}$  (>75%) was observed for seed yield related traits like M: F ratio, 100SW and TSY respectively. However, comparatively low  $h^2_{bs}$  (<72%) was detected for plant growth related traits like PH, CD and CS. High estimates of heritability for M: F ratio (88.07%), 100SW (82.19%) and TSY (75.01) also revealed that environment is less influential on seed yield related traits. Therefore, from the present study it can be inferred that, after gestation period, the selection of elite *J. curcas* plants on the basis of M: F ratio, 100SW and TSY ( $h^2_{bs} > 75\%$ ) for tree improvement and breeding programs will be more successful.

## Conclusion

This study reflected a high level of morphometric variation among 33 *J. curcas* populations. Of these, based on the morphometric traits, IITJC7 (highest 100SW), IITJC15 (highest TSY and minimum M:F ratio), IITJC24 (highest PH and maximum CS) and IITJC28 (maximum CD) were identified as exceptional. Cluster and principal component analyses demarcated IITJC15, IITJC19, IITJC21, IITJC22 and IITJC24 as promising and diverse populations. The seven groups from North-East India (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) identified in this investigation on

the basis of morphometric trait values and cluster analysis results can be recommended as potential starting materials in tree breeding programs for the development of genetically diverse *J. curcas* genotypes with desirable agronomic traits. It is also suggested that during future tree evaluation programs, growth attributes like PH, CS and CD can be used for preliminary screening of young *J. curcas* plants from large scale plantations. However, after gestation period, once the plants start giving economic yields, further screening and selection of agronomically promising plants on the basis of male to female flower ratio, 100 seed weight and total seed yield is likely to be more effective.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

## Effect of Brazil nut oil (*Bertholletia excelsa* HBK) on the physical, chemical, sensory and microbiological characteristics of a mayonnaise-type emulsion

Cristina Grace de Sousa Guerra<sup>1</sup>, Jaime Paiva Lopes Aguiar<sup>2</sup>, Wallice Luiz Paxiuba Duncan<sup>1</sup>, Ariane Mendonça Kluckzosvki<sup>1</sup> and Francisca das Chagas do Amaral Souza<sup>2\*</sup>

<sup>1</sup>Department of Food Science, Federal University of Amazonas, Manaus, Amazonas (AM), Brazil.

<sup>2</sup>National Institute of Amazonian Research, Coordination of Environment, Society and Health, Food and Nutrition Laboratory, Av. André Araújo, 2936, Aleixo, 69060-001, Manaus, AM, Brasil.

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The objective of this study was to evaluate the influence of emulsifiers on the chemico-physical, colorimetric, microscopic and sensorial properties, and the stability of a mayonnaise-type emulsion prepared with Brazil nut oil (*Bertholletia excelsa* H.B.K). For this purpose, two emulsifiers were used: a soya protein isolate and dehydrated and pasteurised egg yolk as a source of protein. Both formulations had high energy and lipid contents but low mineral and carbohydrate contents. The formulation with egg yolk exhibited higher levels of minerals, such as potassium and calcium, than the emulsion with soya. The mean particle diameter of the Brazil nut-soya emulsion ranged from 8.78 to 24.15  $\mu\text{m}$ , and that of the Brazil nut-yolk emulsion ranged from 0.85 to 22.41  $\mu\text{m}$ , indicating that size directly influences the viscosity of the emulsion. Thus, the Brazil nut-soya emulsion can be characterised as a monodisperse emulsion. The Brazil nut-yolk emulsion was darker, or had lower lightness ( $L^*$ ), compared to the Brazil nut-soya emulsion. The formulation with soya protein was demonstrated to be unsuitable for consumption due to a high microbial load, specifically moulds and yeasts, and was excluded from the sensory evaluation. However, the egg yolk emulsion showed acceptable microbiological parameters according to current legislation. The consumer acceptance means were greater than 6.95 (maximum of 9 on the hedonic scale). Acceptance of the emulsion was also confirmed by purchasing attitude, for which 75% of consumers stated they would purchase the product. Thus, soya protein is not viable for the production of an emulsion with Brazil nut oil, whereas egg yolk is a better emulsifier, which can influence the physicochemical, nutritional and sensory parameters. Additionally, the product can be stored at room temperature, which is an economically feasible feature for the consumer market.

**Key words:** Brazil nut, egg yolk emulsion, soya emulsion, mayonnaise, nutritional properties.

### INTRODUCTION

In the Amazon, a wide variety of foods and medicinal plants exist that have different biological properties, many of which have been rarely or never studied. This is

associated with the cultural diversity of the traditional communities of the region, is a treasure for Brazilian biotechnology. The proper characterisation of this

potential can guide actions for sustainable development in the Amazon (Souza et al., 2008). The region, with its wealth of plant species, is known for producing vegetable oils with unique aromas and tastes. The properties of these vegetable oils have been intensively researched, mainly by international companies, due to their various applications in the food, pharmaceutical and other industries (Pardaul et al., 2007). The Brazil nut (*Bertholletia excelsa* Humb. & Bonpl. Lecythidaceae) is considered one of the most important species with economic exploitation of the Amazon rainforest. Almonds are much appreciated for human consumption due to their high nutritional value and health benefits (Massi et al., 2014).

The Brazil nut (*B. excelsa* H.B.K) is one of the most important extractively exploited non-timber forest products and is native to the Amazon region (Freitas-Silva and Venancio, 2001). The production and extraction of the Brazil nut is a low-environmental-impact activity (Wadt et al., 2005). The nuts are oily, with a high energy value, and are rich in proteins of high biological value. As such, they are considered a good alternative source of nutrients in food fortification and an excellent addition to the diet of children and adults because of the high content of lipids, vitamins, minerals, and proteins (Funasaki et al., 2013). The oil extracted from the nuts has good digestibility, and the extraction residue can be used in foods and animal feed (Pacheco and Scussel, 2006).

The concentration of lipids in the Brazil nut is approximately 66%, and the main fatty acids are linoleic (45%), oleic (29%), palmitic (15%), and stearic (10%) (Kornsteiner et al., 2006; Venkatachalam and Sathe, 2006). Balbi et al. (2014) found that Brazil nuts are an important source of fatty acids, proteins, fibers, minerals and selenium. The results obtained by the physicochemical analyses carried out in *B. excelsa* oil are within the parameters established by the Brazilian legislation; the acidic characteristics, the high degree of unsaturation, indicated that the oil contains polyunsaturated fatty acids to a large extent (Pena Muniz et al., 2015).

Lipids are part of the matrix of many food products, such as emulsions. They modify the physical properties of foods, including the flavour, appearance and structure. It has been reported that lipids influence flavour perception in terms of both aroma release and textural changes (Daget et al., 1987; Malone and Appelqvist, 2003). As a result, the reformulation of flavours consisting of foods with reduced fat requires considerable work so that they can meet the needs and expectations of consumers (Rabe et al., 2003). Considering these aspects, the lipids present in the Brazil nut have

enormous potential for the formulation of mayonnaise-type emulsions, in addition to having good nutritional characteristics. However, the physical properties and chemical stability of the emulsions must be studied.

In general, mayonnaise is a semi-solid emulsion of oil in water containing 70 to 80% fat. It is traditionally prepared by carefully mixing egg yolk, vinegar, oil and spices (especially mustard). Largely influenced by concerns about health, there has been pressure on the food industry to reduce the amount of fat, sugar, cholesterol, salt and certain additives in the diet (Liu et al., 2007).

Therefore, the objective of the present study was to investigate the influence of emulsifiers on the chemico-physical, colorimetric, microscopic and sensorial properties, and the stability of mayonnaise-type emulsions made with Brazil nut oil.

## MATERIALS AND METHODS

### Emulsion preparation

Two emulsifiers were used with different protein sources: soya isolate and dehydrated and pasteurised egg yolk. For the preparation of emulsions, the ingredients were pre-weighed in a Filizola analytical balance. The dry ingredients (soya protein isolate (Levlife) or pasteurised and dehydrated egg yolk (Salto's)), salt, and guar gum (Maxfoods) were mixed, and then 1/3 of the Brazil nut oil was added, and the ingredients were homogenised for 5 min in a mixer. Next, vinegar and the remaining oil were added, followed by homogenisation for 2 min (Nikzade et al., 2012).

### Physicochemical characterisation

For proximate composition, moisture was determined by gravimetry in a conventional oven at 105°C to a constant weight. Protein content was determined by the Kjeldahl method, and lipid content was estimated by direct extraction in a Soxhlet apparatus, using petroleum ether as the solvent. The ashes were quantified using gravimetry by incinerating the sample in a muffle furnace at 550°C. Carbohydrate content was calculated by the difference method, by subtracting from 100 the sum of the moisture, protein, lipid and ash contents, following the methodology of the Adolfo Lutz Institute (Instituto Adolfo Lutz, 2008).

### Minerals

Mineral content was determined in triplicate by atomic absorption spectrometry as recommended by the Adolfo Lutz Institute (IAL, 2008) and according to the Varian manual (2000). Samples were digested in a MARS Xpress microwave digester (CEM Corporation, Model – 2591) in organic matter with the use of concentrated nitric acid. The reading was performed directly in dilute solutions in an atomic absorption spectrophotometer (Spectra AA, model 220 FS, Varian, 2000). The following mineral elements were quantified: Ca, K, Na, Mg, Fe, Zn, Mn and Cu. Controls for the analysis followed the recommendations of Cornelis (1992), with the certified reference

\*Corresponding author. E-mail: francisca.souza@inpa.gov.br. Tel: +55 92 3634-1964.

material Peach leaves (NIST-SRM 1547).

### Antioxidant activity of the emulsion

Antioxidant activity was evaluated using the free radical-scavenging assay of the reaction of DPPH (2,2-diphenyl-1-picrylhydrazyl) in absolute ethanol (2 mg DPPH in 12 mL of ethanol). Emulsion test solutions were prepared in various concentrations: 0, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000  $\mu\text{g/mL}$ . Thereafter, 30  $\mu\text{L}$  of the samples was added to the microplate wells. Next, 270  $\mu\text{L}$  of DPPH solution or ethanol was added to the test samples or blanks, respectively. The plates were incubated in the dark for 30 min. Readings were taken at 492 nm in a microplate reader (Multimode Detector DTX 800, Beckman Coulter). The ability to eliminate the DPPH radical (% antioxidant activity) was assessed following the method of Molyneux, 2004.

### Microbiological analysis

The microbiological analysis methods were adopted from the Compendium of Methods for the Microbiological Examination of Foods by the American Public Health Association (APHA, 2001). Total coliforms, *Salmonella* spp, moulds and yeasts were analysed. The analyses were performed on a qualitative basis; the results are expressed as the presence or absence of these microorganisms in 25 g of food.

### Microscopic analysis

Emulsion patterns were analysed using a Nikon E-800 microscope (Kawasaki, Japan) with bright-field illumination at 40 $\times$  magnification. The emulsions were observed after 24 h of cooling at 4°C. For this purpose, 4.0 mL of the emulsion was trickled with a microsyringe over a drop of water (3 mL), previously deposited onto a slide (76  $\times$  26 mm), and covered with a coverslip (24  $\times$  32 mm). Digital images were acquired with a Nikon DXM-1200 camera. Particle sizes were measured from the previously calibrated images (Poyato et al., 2013).

### Colour analysis

The colour of the emulsion was measured in the  $L^*$ ,  $a^*$ ,  $b^*$  system using a spectrophotometer (Mini Hunter Lab Scan XE Plus, Model 45/0-G), which was calibrated using a black and white porcelain plate. For direct reading, the samples were added in a sufficient amount to cuvettes. In this colour system,  $L^*$  represents the changes in lightness, ranging from 0 (black) to 100 (white), and  $a^*$  and  $b^*$  are the colour coordinates responsible for the chromaticity, where  $-a^*$ =green and  $+a^*$ =red and  $-b^*$ =blue and  $+b^*$ =yellow (HunterLab, 2001).

### Shelf life

The shelf life of the emulsion was established using packaging that prevents contact between food and oxygen for seven different time periods (0, 30, 60, 90, 120, 150 and 180 days), during which the samples were stored at room temperature.

### Sensory analysis

Sensory tests were performed in individual booths, illuminated with white light. Sensory analysis of the emulsions was performed using

the global acceptance and purchasing attitude tests. Two samples of approximately 10 g each were served refrigerated ( $10^\circ\text{C} \pm 1^\circ\text{C}$ ), individually, in disposable cups (50 mL) with a plastic spatula, and coded with three random digits. To cleanse the palate, water and salt biscuits were served.

The acceptance test used a hedonic scale applied to 68 untrained judges, aged 22–45 years. A structured nine-point scale was used, where 1 corresponded to "disliked extremely", and 9 to "liked extremely". Purchasing attitude was assessed with the sample that obtained better acceptance in the hedonic scale, using a five-point scale, where 1 corresponded to "I would never purchase this product" and 5 to "I would certainly purchase this product".

### Data analysis

Data are presented as the mean  $\pm$  standard deviation. Data were submitted to a test of normality. To compare each characteristic of the Brazil nut emulsion with egg yolk or soya protein, Student's *t* test or Mann-Whitney U test was used, depending on the normality test. In all cases, the significance level was 5% ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

### Physical, chemical and mineral characterisation of emulsions

The results of the physicochemical parameters of moisture, protein, lipid, ash, carbohydrate and energy are shown in Table 1. It is observed that the values of the above parameters are similar in the Brazil nut–soya (BS) and Brazil nut–dehydrated egg yolk (BY) emulsions, with the exception of the energy value and calcium and potassium contents, which are higher in the BY emulsion. Additionally, it was found that the BS and BY emulsions have more proteins, lipids, energy and minerals than a traditional mayonnaise emulsion (TACO, 2006).

The energy values of the two Brazil nut emulsions (492–597 kcal 100  $\text{g}^{-1}$ ) were low compared to the values estimated for commercial mayonnaise. Traditional mayonnaise has, on average, 680 kcal 100  $\text{g}^{-1}$ , whereas the low-fat versions (light) have 340 kcal 100  $\text{g}^{-1}$  (USDA, 2002). According to TACO (2006), traditional industrialised mayonnaise with eggs has, on average, 302 kcal 100  $\text{g}^{-1}$ .

The emulsions studied are an important source of minerals. These minerals perform essential functions in the body as components of prosthetic groups in proteins or ions dissolved in body fluids that regulate the activities of many enzymes and maintain the acid–base balance and osmotic pressure necessary for physiological homeostasis (Andrade et al., 2003). The minerals found at higher concentrations were sodium, calcium and potassium. Calcium and potassium are important for neuromuscular and muscle activity, bone tissue, cell growth, intracellular homeostasis and hormonal function regulation. Deficiency in these minerals may result in osteoporosis in adults and even rickets in children (Shils et al., 2003).

**Table 1.** Proximate and mineral composition of Brazil nut–soya (BS) and Brazil nut–egg yolk (BY) emulsions.

Mineral composition	BS emulsion	BY emulsion	Traditional*
Moisture (% wb#)	40.41	31.99	58
Protein (%)	6.12	9.6	1
Lipids (%)	51.22	55.84	30
Ash (%)	0.69	1.01	2.6
Carbohydrates (%)	1.55	1.55	8
Total energy (kcal <sup>-1</sup> g)	491.98	547.16	302
Calcium (mg)	15.30	38.71	3.0
Magnesium (mg)	4.30	4.41	1
Potassium (mg)	11.4	62.05	16
Sodium (mg)	870.48	710.48	787
Manganese (mg)	0.30	0.30	<LQ
Copper (mg)	Nd	Nd	Na
Zinc (mg)	0.11	Tr	Na
Iron (mg)	2.62	0.94	0.1

# wb = wet basis. \* Traditional mayonnaise, as in TACO (2006). Tr, Traces; Na, not analysed; Nd, not detected; <, LQ below the limit of quantification.

**Table 2.** Antioxidant activity of Brazil nut–soya protein (BS) and Brazil nut–egg yolk protein (BY) emulsions. The BS and BY values are negative. Gallic acid was used as a standard.

Samples	Mean
BS (10 mg mL <sup>-1</sup> )	-2.12396±1.42
BY (10 mg mL <sup>-1</sup> )	-2.12366±1.42
Gallic acid (10 mg mL <sup>-1</sup> )	86.22544

### Antioxidant activity

The antioxidant activity assays show that none of the emulsions played an antioxidant role detectable by the DPPH assay (Table 2). These data were compared to gallic acid, which was used as a standard. All values were negative, indicating no antioxidant activity. However, lipid peroxidation is the main cause of the deterioration of fatty bodies and is responsible for modifying the odour and flavour of food, as well as the loss of nutritional quality, resulting in depreciation and/or rejection by the consumer (Silva et al., 1999).

### Microbiological analysis

A microbiological evaluation of the emulsions was performed over six months. Microbes were detected only in the Brazil nut–soya protein (BS) emulsion, where high contamination by yeasts and moulds was observed at the beginning of the experiment. The Brazil nut oil–dehydrated and pasteurised egg yolk (BY) emulsion did

not present a microbiological risk because the limits conformed to those specified in current legislation (Table 3). Reis et al. (2014) the storage conditions of nuts seem to have an important influence on the population of fungi *Aspergillus section Flavi*.

### Size of emulsion particles

The Brazil nut–soya protein (BS) emulsion produced larger and more uniform droplets than the Brazil nut–egg yolk protein (BY) emulsion. Thus, according to Worrasinchai et al. (2006), the BS emulsion can be classified as a monodisperse emulsion (uniform droplet size), in contrast to the BY emulsion. The mean particle diameter of the BS emulsion ranged from 8.78 to 24.15 µm, whereas that of the BY emulsion ranged from 0.85 to 22.41 µm, indicating that size directly influences the emulsion. The microscopic images (Figure 1) of the two emulsions confirm the suitable structure for an oil-in-water emulsion.

### Colour analysis

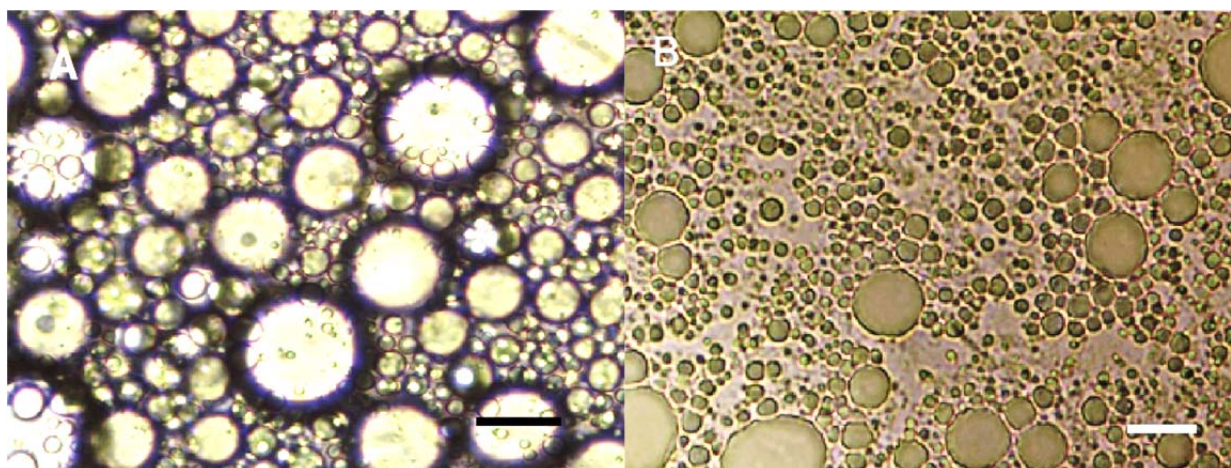
The L parameter represents how light or dark the sample is, and it ranges from 0 (very dark) to 100 (very light) (Bonagurio et al., 2003). The results obtained for colour (Table 4) show that the BY emulsion had a lower lightness values (L\*) than the BS emulsion, indicating that the BY emulsion is darker due to the egg yolk formulation, in contrast with the BS emulsion with soya protein, which had a lighter colour. All of the mayonnaise samples tended more towards white than black because



**Table 3.** Microbiological analyses of Brazil nut oil–soya protein (BS) and Brazil nut oil–dehydrated egg yolk (BY) emulsion samples.

Microbiological parameter	BS emulsion	BY emulsion
Total coliforms	0.0 NMP/g	0.0 NPM/g
Coliforms at 45°C*	0.0 NMP/g	0.0 NMP/g
<i>Staphylococcus aureus</i> *	<10 UFC/g	<10 UFC/g
<i>Salmonella</i> spp.*	Absent	Absent
Yeasts and moulds	11x10 <sup>4</sup> UFC/g	<10 UFC/g

\*Following the Board of Directors Resolution n° 12 of January 2001/Brazilian National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária – ANVISA)/Ministry of Health (Brasil, 2001).



**Figure 1.** (A) Emulsion with Brazil nut oil and soya protein (BS). (B) Emulsion with Brazil nut oil and dehydrated egg yolk (BY). The scales on the images correspond to 20  $\mu$ m.

**Table 4.** Colour parameters L\*, a\*, b\* in Brazil nut oil–soya protein (BS) and Brazil nut oil–dehydrated egg yolk (BY) emulsions.

Samples	L*	a*	b*
BS Emulsion	71.68	2.53	24.52
BY Emulsion	70.48	0.33 <sup>§</sup>	13.59 <sup>§</sup>

<sup>§</sup>Indicates significant difference (Student's t test, P <0.05) between the two types of emulsions.

all produced values above 60, which differed significantly between the two different emulsions. Similar values were found by Pereira et al. (2013), who studied the ideal profile of commercial mayonnaise. Differences existed for the parameters a\* and b\* among the samples. The a\* parameter corresponds to the scale from green to red, where a is negative for green, and a is positive for red; the b\* values correspond to the scale from blue to yellow, where b is negative for blue and b is positive for yellow, according to Bonagurio et al. (2003). Thus, all of the emulsion samples tended to red because all values found

for a\* were positive.

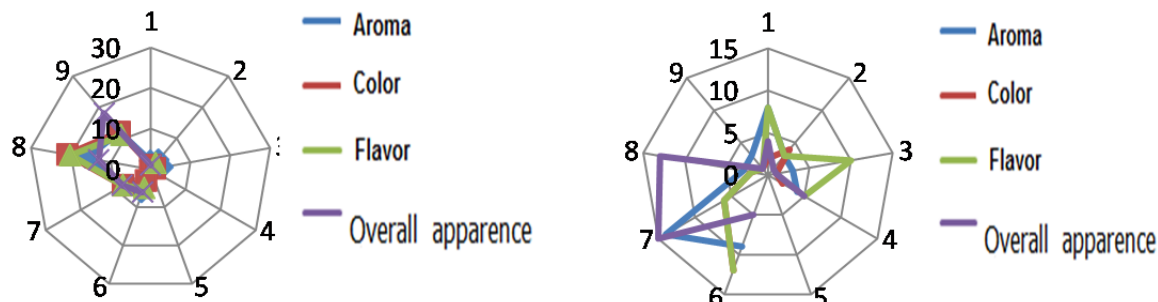
In general, the emulsions exhibited yellow pigmentation because all values were positive, which is a characteristic inherent to this type of product.

The main factors that cause industrial mayonnaise to be yellow are egg yolk, mustard and dyes (Dickinson and Miller, 2001). The colour of egg yolks is attributed to the carotenoids xanthophylls, lutein, zeaxanthin,  $\beta$ -cryptoxanthin and  $\beta$ -carotene, which are solubilised in the yolk (by Li-Chan and Kim 2008).

### Sensory analysis

The results of the sensory analysis acceptance test for aroma, flavour, colour and overall appearance of the emulsion samples are presented in Figures 2. The mean scores obtained for the overall appearance lie in the acceptance zone of the graph (scores of 7 and 8) and, on the hedonic scale, correspond to "liked slightly" and "liked moderately". The colour attribute obtained a score of 8 on the hedonic scale, corresponding to "liked moderately".





### Commercial Emulsion

### BY Emulsion

**Figure 2.** Representation of the acceptance of the commercial emulsion and Brazil nut oil–dehydrated egg yolk (BY) emulsions scored on a hedonic scale. The formulation with soya protein (BS) was determined to be unsuitable for consumption due to the high load of *Bacillus cereus* and was excluded from the sensory analysis.

This may be associated with the egg yolk, which provides a favourable colour to the emulsion. A score of 7 was obtained for aroma, corresponding to "liked slightly", with no reports of distinct odour in the product due to the addition of Brazil nut oil. The flavour attribute was the parameter with the lowest score on the hedonic scale (6), corresponding to "neither liked nor disliked", and no attribute evaluated was rejected.

### Conclusions

Soya protein is not viable for the production of an emulsion with Brazil nut oil. In contrast, egg yolk was a suitable emulsifier. The physicochemical, nutritional and sensory parameters were influenced by the emulsion and also because the product was stored at room temperature, storage makes it economically feasible for the consumer market.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

## Phenolic compounds and antioxidant activity of red and white grapes on different rootstocks

Marlon Jocimar Rodrigues da Silva<sup>1\*</sup>, Bruna Thaís Ferracioli Vedoato<sup>1</sup>, Giuseppina Pace Pereira Lima<sup>2</sup>, Mara Fernandes Moura<sup>3</sup>, Giovanni Marcello de Angeli Gilli Coser<sup>1</sup>, Charles Yukihiro Watanabe<sup>1</sup> and Marco Antonio Tecchio<sup>1</sup>

<sup>1</sup>Faculdade de Ciências Agrônomicas, Universidade Estadual Paulista (UNESP), Câmpus Botucatu, Rua José Barbosa de Barros, 1780, CEP 18610-307, Botucatu-SP, Brasil.

<sup>2</sup>Universidade Estadual Paulista (UNESP), Instituto de Biociências, Distrito Rubião Junior, s/n, CEP 18618-970, Botucatu-SP, Brasil.

<sup>3</sup>Centro APTA de Frutas, Instituto Agrônomico de Campinas, Avenida Luiz Pereira do Santos, 1500, CEP 13.214-820, Jundiá, SP, Brasil.

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This study aimed to assess the rootstocks influence over total phenolic compound content, antioxidant activity and its correlation in different red and white grapes cultivars for wine production. This study conducted an experimental three-year-old vineyard, located in Jundiá, in the State of São Paulo, Brazil, from July 2013 to January 2014. Red and white grapes from *Vitis vinifera* L. (Cabernet Sauvignon, Cabernet Franc, Merlot, Syrah and Sauvignon Blanc), *V. labrusca* L. (Isabel and Bordô) and hybrid cultivars (IAC 138-22 Máximo, BRS Violeta, IAC 116-31 Rainha, IAC 21-14 Madalena and BRS Lorena) were grafted on IAC 766 and 106-8 Mgt rootstocks. Samples of grapes were collected and the total carotenoids, chlorophyll, anthocyanin, flavonoid, and phenolic content, and the *in vitro* antioxidant activity determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The rootstocks effects on red grape were mainly observed on the anthocyanins content. The total polyphenols content and the grapes antioxidant activity was virtually not affected by the rootstocks, especially on grapes of BRS Violeta and IAC 138-22 Máximo kinds, which presented the highest content of this compost. Among the white grapes, the 106-8 Mgt rootstock favored the increase of total phenolic compounds content on grapes of Sauvignon Blanc, IAC 116-31 Rainha and IAC 21-14 Madalena kinds. Although the hybrid grapes of IAC 116-31 Rainha e IAC 21-14 Madalena kinds are white grapes, the total phenolic compounds content on them were higher than the ones found on the red grape Isabel.

**Key words:** *Vitis vinifera*, *Vitis labrusca*, hybrid grapes, total anthocyanin, Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH).

### INTRODUCTION

The composition and phenolic properties of grapes, especially those aimed for wine and juice production, have been constantly studied, and there are more and

more reports of higher amounts of phenolic compounds acting as antioxidants on grapes (Rockenbach et al., 2011). However, most data available in literature about

phenolic compounds in grapes and wines come from traditionally producing countries, mainly from Europe, where the grapes of choice are mostly *Vitis vinifera*. In contrast, in Brazil, more than 85% of the processed grapes volume comes from American cultivars, mainly *Vitis labrusca*, or hybrids, these kinds being considered more fitting to the climate conditions of the country, especially during harvesting season in the South and Southeast Regions of Brazil (Lago-Vanzela et al., 2011).

The *V. labrusca*, Isabel and Bordô grape cultivars are widely farmed in Brazil and, as a way of diversifying the products giving the producers more options, the Campinas Agronomic Institute (Instituto Agronômico de Campinas - IAC) and the Brazilian Company of Farming Research (Empresa Brasileira de Pesquisa Agropecuária - Embrapa), along with their respective genetic improvement programs, have been developing in the past few years new hybrid cultivars for wine production (Biasoto et al., 2014; Burin et al., 2014; Lago-Vanzela et al., 2013), among others there are the IAC 138-22 'Máximo' ('Seibel 11342' x 'Syrah'), IAC 116-31 'Rainha' ('Seibel 7053' x 'Burgunder Kastenholtz'), IAC 21-14 'Madalena' ('Seibel 11342' x 'Moscatel de Canelli'), 'BRS Lorena' ('Malvasia Bianca' x 'Seyval') and the 'BRS Violeta' ('BRS Rúbea' x 'IAC 1398-21') grape cultivars. It is known that these varieties present high producing capability and low sensibility to the main fungal diseases that usually attack grapevines. However, there is lack of information about these grapes phenolic compounds and antioxidant activity.

Several studies show that phenolic compounds in grapes may vary due to some specific factors, such as the species, cultivar, climate conditions, geographic region and the grapevines handling practices (Barcia et al., 2014; Burin et al., 2014; Koundouras et al., 2009). On the other hand, studies assessing the rootstocks influence over phenolic compounds content and antioxidant activity, especially on grapes for wine production, are scarce. Some studies have demonstrated that weaker rootstocks enable higher concentrations of anthocyanin and phenolic compounds to accumulate on grapes peel. There were no effects of 1103 Paulsen (*Vitis rupestris* x *Vitis berlandieri*) and SO4 (*Vitis riparia* x *V. berlandieri*) rootstocks over the Cabernet Sauvignon cultivars' anthocyanin content and total phenolic compounds (Koundouras et al., 2009). However, these compounds were strongly influenced on Bordô cultivars, when the cultivar was grafted in different rootstocks (Mota et al., 2009).

Generally, rootstocks are recommended based on, to better fit to, environmental conditions and cultivar compatibility, what directly affects productivity and some of the fruits chemical characteristics, such as the pH,

acidity and soluble solids content. However, the intake of nutrients, phenolic compounds concentration and anthocyanin content are quality parameters that should be taken into account when farming grapes, mainly, to better choose the most efficient combination of cultivar and rootstock (Mota et al., 2009).

Therefore, this study is aimed at assessing the rootstocks influence over phenolic compounds content, antioxidant activity and their correlation in different red and white grapes cultivars for wine production.

## MATERIALS AND METHODS

### Experimental site and grapevines growing conditions

This study was conducted in an experimental 3-year-old vineyard, located in Jundiaí (23° 06' S, 46° 55' O, altitude 745 m) in the State of São Paulo (SP), Brazil, from July 2013 to January 2014. According to the Köppen classification, the climate is type *Cfb*, that is, subtropical climate with an average temperature of 19.5°C, and the average annual rainfall rate is of 1400 mm, with a tendency for concentrated rainfall during the summer months. The soil of the area was classified as Red Cambisol according to previously published criteria (EMBRAPA, 2006).

The vines were supported by trellising system and spaced 2.5 x 1.0 m apart (4000 vines ha<sup>-1</sup>). Pruning was performed leaving one bud behind in each of them. Subsequently 5% hydrogen cyanamide was applied on the buds to induce and standardize sprouting. At the changing color stage of berries, plants were protected with anti-hail screens, aiming to protect them against hailstorms, and attack by birds and bees.

### Plant material and samples preparation

Red and white grapes from *V. vinifera* L. (Cabernet Sauvignon, Cabernet Franc, Merlot, Syrah and Sauvignon Blanc), *V. labrusca* L. (Isabel and Bordô) and hybrid cultivars (IAC 138-22 Máximo, BRS Violeta, IAC 116-31 Rainha, IAC 21-14 Madalena and BRS Lorena) were grafted in IAC 766 (106-8 Mgt x *V. caribaea*) and 106-8 Mgt [*V. riparia* x (*V. rupestris* x *V. cordifolia*)] rootstocks. All the grapes cultivars were harvested at the stage of their respective technical maturity, according to their soluble solids contents, titratable acidity and pH (Table 1).

10 clusters for each experimental trial were randomly selected. 10 berries from each cluster (from the clusters' top, middle and bottom), were collected adding up to 100 berries per trial, which then were cut in half and had their seeds removed and frozen in liquid nitrogen, pulverized and stored under -20°C up to the moment of analysis.

### Chemicals

Methyl alcohol and acetone were obtained from Tedia (Fairfield, Ohio, USA). Tris-(hydroxymethyl)-aminomethane and Folin-Ciocalteu reagents were obtained from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,7,8-tetramethylchroman-2-carboxylic acid) and DPPH radical (2,2-Diphenyl-1-picrylhydrazyl) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

\*Corresponding author. E-mail: marlonjocimar@gmail.com.

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**Table 1.** Physicochemical parameters for berries of red and white grape varieties onto different rootstocks.

Cultivars/rootstocks	SS (°Brix)		pH		TA (% tartaric acid)	
	IAC 766	106-8 Mgt	IAC 766	106-8 Mgt	IAC 766	106-8 Mgt
<b>Red cultivars</b>						
Isabel	20.40	18.28	3.30	3.21	1.17	1.14
Bordô	16.76	15.85	3.44	3.49	0.33	0.30
Cabernet Sauvignon	19.68	18.03	3.95	3.82	0.93	1.03
Cabernet Franc	20.68	18.82	4.10	3.97	0.68	0.66
Merlot	20.66	18.13	3.93	3.87	0.67	0.85
Syrah	18.18	17.30	3.85	3.90	0.86	0.88
IAC 138-22 Máximo	17.42	16.00	3.60	3.55	0.90	0.93
BRS Violeta	18.36	18.56	3.55	3.59	0.87	0.79
<b>White cultivars</b>						
Sauvignon Blanc	19.12	19.52	3.18	3.21	0.86	0.88
IAC 116-31 Rainha	20.00	20.82	3.41	3.42	0.90	0.88
IAC 21-14 Madalena	18.82	19.40	3.57	3.50	0.82	0.82
BRS Lorena	22.74	20.50	3.38	3.31	0.77	0.84

SS, Soluble solids content; TA, titratable acidity.

#### Determination of total phenolic compounds and antioxidant activity

##### Total anthocyanins, carotenoids and chlorophyll

The pigments based on the methodology proposed by Sims and Gamon (2002) was determined. The extraction of pigments was done in TRIS-HCl-buffered acetone (80:20 of PA acetone and TRIS 0.2 M) in an environment shielded from light. Afterwards the supernatants and the samples' absorbance were retrieved and measured (663 nm for chlorophyll *a*, 647 nm for chlorophyll *b*, 537 nm for anthocyanin and 470 nm for carotenoids). All of the readings were executed with a BEL Photonics®, SP 2000 UV/vis spectrophotometer. Then, the absorbance results were converted to mg 100 g<sup>-1</sup> of fresh mass.

##### Total flavonoids

So the total flavonoids content could be assessed; the extracts were prepared according to the method described by Popova et al. (2004), slightly altered. The extraction was performed through an acidified methanol solution (85:15 of 70% methanol and 10% acetic acid) and later, by adding a 5% aluminum chloride solution. With a spectrophotometer (BEL Photonics®, SP 2000 UV/vis) an absorbance at 425 nm was measured. The total flavonoids content was then calculated by means of quercetin standard curve and the results presented as mg 100 g<sup>-1</sup> quercetin equivalent of fresh mass.

##### Total phenolics content

The grapes' total phenolic content was determined using the Folin-Ciocalteu colorimetric method (Singleton and Rossi Jr, 1965). The absorbance value at 765 nm was obtained in tests on an UV-Vis SP 2000 BEL Photonics® spectrometer and it was compared with a calibration curve obtained for gallic acid. The results were expressed as mg 100<sup>-1</sup> gallic acid equivalents (GAE).

#### *In vitro* antioxidant activity

The grapes' *in vitro* antioxidant activity was determined using the DPPH radical scavenging method (Brand-Williams et al., 1995; Rossetto et al., 2009). The analytical standard Trolox was used to build the calibration curves and the results were expressed as mg of Trolox equivalents per g of grape (mg TEAC g<sup>-1</sup>). Absorbance measurements were performed on a UV-Vis SP 2000 BEL Photonics® spectrometer and the grapes' antioxidant activity was assessed through the decay rate in absorbance at 517 nm. The DPPH radical (2,2-diphenyl-1-picrylhydrazyl) solution was prepared in 80% methanol. For each sample, the absorbance was determined at t = 60 min after adding DPPH radical.

#### Experimental design and statistical analyzes

The experimental design was a randomized block with 16 treatments for red grapes and 8 treatments for white grapes, both with four replications. All analyses were performed in triplicate. Red and white grapes were assessed separately. The data were subjected to variance analysis, and when this assessment indicated statistically significant treatment effects, the data were subjected to the Scott-Knott means comparison test ( $p < 0.05$ ), aided by the SISVAR statistical program (Ferreira, 2011). The correlation analysis was also conducted ( $p < 0.05$  and  $p < 0.01$ ) to investigate the relation between the total phenolic compounds content and antioxidant activity, aided by the ASSISTAT statistical program (Silva and Azevedo, 2002).

## RESULTS

The total carotenoid content among red grapes was higher for the BRS Violeta cultivar in 106-8 Mgt rootstocks, followed by the BRS Violeta cultivar in IAC 766 rootstocks, with respective results of 7.59 and 6.48 mg 100 g<sup>-1</sup> (Table 2). Regarding the Cabernet Sauvignon

**Table 2.** Pigments, total phenolic compounds and antioxidant activity of red grapes on different rootstocks.

Rootstock cultivar	Total carotenoids (mg 100 <sup>-1</sup> g)	Total anthocyanin (mg 100 <sup>-1</sup> g)	Total flavonoids <sup>1</sup> (mg 100 <sup>-1</sup> g)	Total phenolics <sup>2</sup> (mg 100 <sup>-1</sup> g)	Antioxidant activity <sup>3</sup> (mg g <sup>-1</sup> )
<b>IAC 766 Rootstock</b>					
Isabel	0.62±0.05 <sup>d</sup>	3.63±0.35 <sup>e</sup>	11.15±1.14 <sup>d</sup>	294.3±31 <sup>d</sup>	2.13±0.20 <sup>d</sup>
Bordô	0.95±0.10 <sup>d</sup>	6.00±0.85 <sup>e</sup>	19.51±2.36 <sup>b</sup>	545.2±34 <sup>b</sup>	5.60±0.71 <sup>b</sup>
Cabernet Sauvignon	1.37±0.32 <sup>c</sup>	8.72±2.16 <sup>d</sup>	12.87±1.36 <sup>d</sup>	501.7±38 <sup>b</sup>	4.91±0.88 <sup>c</sup>
Cabernet Franc	1.96±0.26 <sup>c</sup>	12.92±2.00 <sup>c</sup>	17.55±3.60 <sup>c</sup>	481.2±99 <sup>b</sup>	4.77±0.57 <sup>c</sup>
Merlot	1.13±0.03 <sup>c</sup>	7.25±0.27 <sup>e</sup>	15.09±1.69 <sup>c</sup>	386.9±43 <sup>c</sup>	4.03±0.22 <sup>c</sup>
Syrah	0.65±0.09 <sup>d</sup>	3.79±0.68 <sup>e</sup>	12.51±1.59 <sup>d</sup>	414.1±91 <sup>c</sup>	4.28±0.99 <sup>c</sup>
IAC 138-22 Máximo	1.69±0.45 <sup>c</sup>	13.88±2.31 <sup>c</sup>	26.93±6.64 <sup>a</sup>	659.6±99 <sup>a</sup>	6.44±1.50 <sup>a</sup>
BRS Violeta	6.48±0.87 <sup>b</sup>	45.80±5.49 <sup>b</sup>	27.08±2.26 <sup>a</sup>	714.3±183 <sup>a</sup>	7.61±1.12 <sup>a</sup>
<b>106-8 Mgt Rootstock</b>					
Isabel	0.53±0.08 <sup>d</sup>	2.41±0.24 <sup>e</sup>	9.58±2.35 <sup>d</sup>	287.7±41 <sup>d</sup>	2.11±0.50 <sup>d</sup>
Bordô	1.00±0.08 <sup>d</sup>	6.19±0.49 <sup>e</sup>	20.31±2.19 <sup>b</sup>	523.0±64 <sup>b</sup>	5.38±0.74 <sup>b</sup>
Cabernet Sauvignon	0.47±0.03 <sup>d</sup>	2.54±0.17 <sup>e</sup>	10.41±1.51 <sup>d</sup>	474.4±77 <sup>b</sup>	4.76±0.49 <sup>c</sup>
Cabernet Franc	1.28±0.27 <sup>c</sup>	6.44±0.78 <sup>e</sup>	14.51±4.89 <sup>c</sup>	438.4±46 <sup>b</sup>	5.18±0.31 <sup>b</sup>
Merlot	0.74±0.26 <sup>d</sup>	4.28±1.79 <sup>e</sup>	11.17±2.84 <sup>d</sup>	419.0±66 <sup>c</sup>	3.84±0.73 <sup>c</sup>
Syrah	0.69±0.05 <sup>d</sup>	3.84±0.35 <sup>e</sup>	10.50±0.86 <sup>d</sup>	398.0±56 <sup>c</sup>	3.47±0.89 <sup>c</sup>
IAC 138-22 Máximo	1.43±0.06 <sup>c</sup>	8.81±0.38 <sup>d</sup>	21.62±5.35 <sup>b</sup>	669.9±87 <sup>a</sup>	6.63±1.15 <sup>a</sup>
BRS Violeta	7.59±1.34 <sup>a</sup>	54.12±9.96 <sup>a</sup>	28.20±2.55 <sup>a</sup>	722.3±119 <sup>a</sup>	7.41±1.09 <sup>a</sup>
CV (%)	23.69	24.78	18.96	17.20	16.85

Means values ± standard deviation followed by different letters in the same column differ significantly (Scott-Knott test,  $p < 0.05$ ). <sup>1</sup>Total flavonoids expressed as mg 100<sup>-1</sup> g equivalent to quercetin. <sup>2</sup>Total phenolics measured with Folin-Ciocalteu expressed as mg 100<sup>-1</sup> g equivalent to gallic acid. <sup>3</sup>Antioxidant activity expressed mg equivalents of Trolox g<sup>-1</sup>.

and Merlot cultivars, the total carotenoid content of grapes grafted in IAC 766 rootstocks was higher than the ones obtained with the same cultivars in 106-8 Mgt rootstocks.

As well as the results regarding the total carotenoid content, the highest, the highest total anthocyanin content was found in BRS Violeta grapes grafted in 106-8 Mgt rootstocks (54.12 mg 100<sup>-1</sup>), higher than the one obtained by the same cultivar grafted in IAC 766 rootstocks (45.80 mg 100<sup>-1</sup>). However, the highest total anthocyanin content in Cabernet Sauvignon, Cabernet Franc and IAC 138-22 Máximo was obtained when these grapevines were grafted in IAC 766 rootstocks.

IAC 138-22 Máximo grafted in IAC 766 rootstock and BRS Violeta in both rootstocks, presented the highest total flavonoid contents (27.40 mg 100 g<sup>-1</sup>). The total flavonoid content obtained from IAC 138-22 Máximo and Merlot grapes in IAC 766 rootstocks was higher than the ones found in the same grapes grafted in 106-8 Mgt rootstocks. There were no rootstock effects over the total phenolic compound content in red grapes. The highest content was found in BRS Violeta and IAC 138-22 Máximo grapes (718.3 and 664.8 mg 100<sup>-1</sup> g, respectively) and these results are 2.38 times higher than the ones obtained from Isabel cultivars (291.0 mg 100<sup>-1</sup>

g), which presented the lowest total phenolic compound content among the red grapes. Similar to the total phenolic compound content, the highest antioxidant activity among red grapes was found in BRS Violeta and IAC 138-22 Máximo cultivars in both rootstocks studied, 7.51 and 6.54 mg g<sup>-1</sup>, respectively, and the lowest antioxidant activity was obtained from Isabel cultivars (2.12 mg g<sup>-1</sup>).

The rootstocks effect over white grapes is shown on Table 3. In both rootstocks studied, grapes from Sauvignon Blanc cultivars presented the highest total chlorophyll content and the lowest total carotenoids content, 424.8 and 0.15 mg 100<sup>-1</sup> g, respectively. There were no rootstocks effect over the total flavonoid content in white grapes and the highest contents were found in IAC 116-31 Rainha and IAC 21-14 Madalena cultivars (3.79 and 3.38 mg 100<sup>-1</sup> g, respectively).

Grapes from IAC 116-31 Rainha cultivars grafted in 106-8 Mgt rootstocks presented the highest total phenolic compound content (511.8 mg 100<sup>-1</sup> g) and the highest antioxidant activity (5.33 mg g<sup>-1</sup>), superior to those obtained from the fruits of the same cultivar when grafted in IAC 766 rootstocks. On the other hand, Sauvignon Blanc cultivars grafted in IAC 766 rootstocks and BRS Lorena cultivars grafted in both rootstocks had the lowest

**Table 3.** Pigments, total phenolic compounds and antioxidant activity of white grapes on different rootstocks.

Rootstock cultivar	Total carotenoids (mg 100 <sup>-1</sup> g)	Total chlorophyll (mg 100 <sup>-1</sup> g)	Total flavonoids <sup>1</sup> (mg 100 <sup>-1</sup> g)	Total phenolics <sup>2</sup> (mg 100 <sup>-1</sup> g)	Antioxidant activity <sup>3</sup> (mg g <sup>-1</sup> )
<b>IAC 766 Rootstock</b>					
Sauvignon Blanc	0.14±0.01 <sup>b</sup>	407.7±52 <sup>a</sup>	1.53±0.12 <sup>b</sup>	236.1±32 <sup>d</sup>	1.54±0.31 <sup>c</sup>
IAC 116-31 Rainha	0.12±0.01 <sup>c</sup>	191.8±25 <sup>c</sup>	4.08±0.68 <sup>a</sup>	444.9±18 <sup>b</sup>	4.37±0.46 <sup>b</sup>
IAC 21-14 Madalena	0.09±0.01 <sup>c</sup>	241.7±18 <sup>b</sup>	3.13±0.58 <sup>a</sup>	368.3±46 <sup>c</sup>	3.62±0.09 <sup>b</sup>
BRS Lorena	0.10±0.01 <sup>c</sup>	223.8±16 <sup>c</sup>	2.20±0.50 <sup>b</sup>	271.8±27 <sup>d</sup>	2.21±0.78 <sup>c</sup>
<b>106-8 Mgt Rootstock</b>					
Sauvignon Blanc	0.16±0.01 <sup>a</sup>	441.8±72 <sup>a</sup>	2.15±0.47 <sup>b</sup>	320.6±65 <sup>c</sup>	2.12±0.47 <sup>c</sup>
IAC 116-31 Rainha	0.10±0.01 <sup>c</sup>	202.1±30 <sup>c</sup>	3.49±0.65 <sup>a</sup>	511.8±76 <sup>a</sup>	5.33±0.71 <sup>a</sup>
IAC 21-14 Madalena	0.11±0.02 <sup>c</sup>	273.1±58 <sup>b</sup>	3.63±0.89 <sup>a</sup>	426.9±41 <sup>b</sup>	3.68±0.60 <sup>b</sup>
BRS Lorena	0.12±0.00 <sup>c</sup>	266.5±26 <sup>b</sup>	1.79±0.66 <sup>b</sup>	293.5±10 <sup>d</sup>	2.17±0.17 <sup>c</sup>
CV (%)	9.62	14.46	21.08	13.05	16.32

Means values ± standard deviation followed by different letters in the same column differ significantly (Scott-Knott test,  $p < 0.05$ ). <sup>1</sup>Total flavonoids expressed as mg 100<sup>-1</sup> g equivalent to quercetin. <sup>2</sup>Total phenolics measured with Folin-Ciocalteu expressed as mg 100<sup>-1</sup> g equivalent to gallic acid. <sup>3</sup>Antioxidant activity expressed equivalents of Trolox g<sup>-1</sup>.

**Table 4.** Pearson's correlation analysis between quantification of phenolic compounds and antioxidant activity of red and white grape cultivars measured by DPPH method.

Cultivars	Antioxidant activity (DPPH)	
	Pearson $r$	$p$ -Values
<b>Red</b>		
Total anthocyanin	0.63	$p < 0.01$
Total carotenoids	0.62	$p < 0.01$
Total flavonoids	0.79	$p < 0.01$
Total phenolics	0.84	$p < 0.01$
<b>White</b>		
Total chlorophyll	-0.63	$p < 0.01$
Total carotenoids	-0.46	$p < 0.01$
Total flavonoids	0.76	$p < 0.01$
Total phenolics	0.91	$p < 0.01$

$p < 0.01$ , Significant correlations at 1% probability of error.

total phenolic compound contents and regardless of the studied rootstock, these cultivars also presented the lowest antioxidant activities among white grapes.

There was significant correlation among all of the assessed compounds and the antioxidant activity (Table 4). The total phenolic compound contents had the highest correlation rates regarding the antioxidant activity, both in red grapes ( $r = 0.84$ ,  $p < 0.01$ ) and white grapes alike ( $r = 0.91$ ,  $p < 0.01$ ). Only in white grapes there was a negative correlation between the total chlorophyll content ( $r = -0.63$ ,  $p < 0.01$ ) and total carotenoids ( $r = -0.46$ ,  $p < 0.01$ ) regarding the antioxidant activity.

## DISCUSSION

Although the total carotenoids content in grapes for wine production was not varied between rootstocks on most cultivars studies, a big difference was found regarding the pigments content both in red (Table 2) and white grapes (Table 3). Despite the importance these pigments exercise over their own by-products, especially on wines, yet studies regarding carotenoid contents in grapes are rare. However, some specific factors, such as grapes cultivar, farming region, the cluster's exposure to sunlight and its maturation period are known to affect carotenoids concentration in grapes (Mendes-Pinto et al., 2005). The carotenoids, along with pyrazines and terpenes, are responsible for wine's primary scent. These pigments' oxidative decay, which occurs while the berry is growing or even during the must's fermentation process, originates norisoprenoids, thirteen-carbon-hydrocarbons that have no immediate influence over wine's sensory characteristics, however, the products from its decay produce volatile compounds important for its aromatic composition (Jackson, 2000; Mendes-Pinto et al., 2005).

The total chlorophyll content found in hybrid grapes was lower than the one found in *V. Vinifera* and Sauvignon Blanc grapes (Table 3). Several factors may be related to the presence and content of such pigments on grapes. The chlorophyll's synthesis and concentration in grape berries is prompted by exposing the cluster to the sun and in the absence of light, chlorophyll's synthesis is then decreased (Downey et al., 2004). The chlorophyll's content in these fruits may still vary due to the characteristics of each different cultivar, the vineyard's environmental condition and the farming practices applied to the grapevines (Kamffer et al., 2010).



Considering that there was a negative correlation between the total chlorophyll content and the antioxidant activity ( $r = -0.63$ ,  $p < 0.01$ ) in white grapes (Table 4), it's possible to infer that the high content of this pigment in these grapes is unwanted. In this study, the highest total chlorophyll content in grapes from Sauvignon Blanc cultivars was found to present fewer antioxidant activities, along with BRS Lorena.

Total anthocyanin content in red grapes suffered a considerable variation among the cultivars of crops studied (Table 2). Among the cultivars, there was no effect from the rootstocks over the anthocyanin content in Isabel, Bordô, Merlot and Syrah cultivars. A Bordô cultivar grafted in both 'IAC 766' and '106-8 Mgt' rootstocks in Caldas (Minas Gerais) has not presented significant difference regarding the anthocyanin content, however these pigments content ( $9.86 \text{ mg g}^{-1}$  in IAC 766 and  $10.47 \text{ mg g}^{-1}$  in 106-8 Mgt) were higher comparing to the ones found in our study, it was likely due to the fact that our data reflect the contents in the whole fruit, whilst the aforementioned authors had assessed the presence of anthocyanin only in peels, where this compound's concentration is high.

The difference between rootstocks regarding total anthocyanin content in Cabernet Sauvignon grapes, grafted in 'IAC 766' rootstocks, differs from other studies performed in different regions (Table 2). In these studies, different rootstocks have not been the cause of any alterations on anthocyanin content and other secondary metabolites within the same cultivar. In Davis (USA) there was no effect from the rootstocks 420A (*Vitis berlandieri* × *V. riparia*) and 110R (*V. berlandieri* × *V. rupestris*) over the total anthocyanin content in Cabernet Sauvignon grapes (Lee and Steenwerth, 2013). Likewise, the total anthocyanin content found in the same cultivar grown in Larissa (Greece) did not differ because of the rootstocks 1103 Paulsen (*V. rupestris* × *V. berlandieri*) and SO4 (*V. riparia* × *V. berlandieri*) (Koundouras et al., 2009). In the assessment of the results, it should be noted that different rootstocks was used and the results are likely related to these plants' inner characteristics, to its vigor, in addition to environmental conditions in the harvest place.

The high anthocyanin content found in BRS Violeta grapes grafted in both rootstocks, IAC 766 ( $45.80 \text{ mg g}^{-1}$ ) and 106-8 Mgt ( $54.12 \text{ mg g}^{-1}$ ), is related to an innate characteristic from the cultivar, which is also described by other authors both in fruits (Barcia et al., 2014), and in wines and juices made from these fruits (Lago-Vanzela et al., 2014; Lima et al., 2014). BRS Violeta grapes are covered by a very thick skin, which contains the highest concentration of phenolic compounds, mainly anthocyanin, predominantly (major compound) anthocyanidin 3,5-diglucoside (Rebello et al., 2013).

Total flavonoid contents have varied among the cultivars, with a higher content among red grapes. This result had already been described in other studies stating

that total flavonoid content in Cabernet Sauvignon and Merlot grapes are higher than the ones found in Sauvignon Blanc grapes (Burin et al., 2014). These authors observed, however, that although it's a white grape cultivar of crop, total flavonoid content found in these grapes was higher than the ones from red grapes cultivars, Isabel and Bordô, differing from our results. It is possible that the anthocyanin contents taken from grapes in the present study have affected positively the high total flavonoid rates found through spectrophotometry, making it necessary for the analyses in successive cycles or even so the use of more sophisticated techniques in order to confirm the results obtained. Significant variations on total flavonoid levels might be attributed to several factors, such as genetic, climate, vineyard handling, grape maturation level, berry size (Rockenbach et al., 2011), extraction method and these compounds assessment alike. The species, cultivar and part of the grape (peel, pulp or seed) assessed yet affects the presence and quantity of grapes main flavonoids (Burin et al., 2014; Rockenbach et al., 2011). It is important to notice that flavonols are the best cofactors for a wine's anthocyanin copigmentation and that the higher the grape's flavonoids content is, the higher is also the amount of anthocyanin transferred into the wine during the wine production process (Schwarz et al., 2005).

There was no effect from rootstocks over total phenolic compounds of red and white grapes. Usually, different rootstocks effects on grapes' phenolic compounds content are likely related to several factors, such as vigor, water and nutrients absorption capability, resistance to diseases and their interaction with the grapevine, possibly affecting directly these plants' primary and secondary metabolites and, consequently, the grapevines' productive characteristics and quality of the grapes (Lee and Steenwerth, 2013; Tecchio et al., 2014). The fact that rootstocks have no effect on the total phenols content in Bordô grapes has already been described in previous studies. When grown in Caldas (Minas Gerais, Brazil), this cultivar's total phenolic compounds content has not suffered any effects from 'IAC 766' and '106-8 Mgt' rootstocks, presenting a total phenols average rate of 11.65 and 12.58  $\text{mg g}^{-1}$  in their peels, respectively (Mota et al., 2009). These authors observed that the contents were higher than the ones found on other studied rootstocks, such as 'IAC 572' and 'RR 101-14' and they came to the conclusion that rootstocks of less vigor prompt a higher concentration of phenolic compounds in grapes' skins.

As the rootstocks 'IAC 766' and '106-8 Mgt' have not affected Cabernet Sauvignon cultivar's total phenols content, other researches demonstrate that some rootstocks have no effects over these grapes' total phenols content. When the 'SO4' and '1103 Paulsen' rootstocks were used in Greece (Koundouras et al., 2009) it was observed that the total phenols content did not differ significantly due to the studied rootstocks

contents of 26.9 mg g<sup>-1</sup> were found in the grape's skin, in both rootstocks. These results are higher than the ones found in the present study along the same cultivars, however, it must be noted that analysis were performed from the grape's skin, where these compounds highest concentrations are found, whilst in the current study both skin and pulp were assessed.

Among all the cultivars studied, the highest total phenol content and antioxidant activity were found in BRS Violeta and IAC 138-22 Máximo grapes. Although determining phenol compounds and antioxidant activity in grapes is important for producers in order to decide which cultivar to choose or even a basis for genetic improvement projects; most studies in literature is aimed at the analysing these compounds and their by-products, especially juices and wines. BRS Violeta cultivars have been widely researched in several Brazilian grape producing regions for industrial purposes due to, among other reasons, the high phenolic compounds content and antioxidant activity presented by this cultivar (Lago-Vanzela et al., 2014; Lima et al., 2014; Rebello et al., 2013), however, few studies are available regarding these compounds content in IAC 138-22 Máximo grapes, which presented results close to those found for BRS Violeta.

High total phenolic compounds content was also found in IAC 116-31 Rainha (511.8 mg 100<sup>-1</sup>) and IAC 21-14 Madalena (426.9 mg 100<sup>-1</sup>) cultivars, when grafted in 106-8 Mgt rootstocks. Consequently, these grapes antioxidant activity was high (5.33 and 3.68 mg g<sup>-1</sup>, respectively). Even though these grapes are white, the phenolic compounds content, as well as the antioxidant activity found in these cultivars were higher than those from Isabel red grapes, which contain 291 mg 100<sup>-1</sup> of total phenols and 2.12 mg g<sup>-1</sup> of antioxidant activity taken via DPPH. Isabel grapes are one of the main Brazilian grapes for juice and wine production, and many other studies present these compounds have low content in the fruits, as well as in juices (Burin et al., 2014; Nixford and Herмосín-Gutiérrez, 2010). In spite of the results found and the possibility of producing white wines with high total phenol content and antioxidant activity equivalent or superior to those of red wines, studies involving IAC 116-31 Rainha and IAC 21-14 Madalena grapes are limited in literature.

Regarding antioxidant activities, many studies have reported difficulties in obtaining similar data among grapes, making it difficult to compare results, due to factors such as the use of different analytic methods (DPPH, ABTS, FRAP, ORAC, among others), norms and unities of measurement or even due to the differences in reference materials taken from grapes (the whole grape; skin, pulp or seeds individually, bagasse) (González-Centeno et al., 2013; Lago-Vanzela et al., 2011).

Besides total phenolic compounds ( $r = 0.84$ ,  $p < 0.01$ ), other compounds found in red grapes such as total anthocyanin ( $r = 0.63$ ,  $p < 0.01$ ), total carotenoids ( $r =$

$0.62$ ,  $p < 0.01$ ) and total flavonoids ( $r = 0.79$ ,  $p < 0.01$ ) present positive correlation to antioxidant activity. This is in accordance with many authors who have also observed positive correlation between total phenols and antioxidant activity in red grapes (Burin et al., 2014; Rockenbach et al., 2011; Xu et al., 2010). On the other hand, a few other studies demonstrated no correlations while others presented a negative correlation between these variables (Nixford and Herмосín-Gutiérrez, 2010), which means variation is due to different cultivars, rootstocks and farming sites. Among white grapes, the antioxidant activity is correlated positively to flavonoids ( $r = 0.76$ ,  $p < 0.01$ ) and, mainly to total phenols ( $r = 0.91$ ,  $p < 0.01$ ). A positive correlation between phenol contents and antioxidant activity has also been demonstrated by González-Centeno et al. (2013) in white grapes Chardonnay, Macabeu, Parellada and Premsal Blanc; all the *V. vinifera* were grown along the Balearic Islands, Spain.

## Conclusions

In conclusion, the farming of red grapes Cabernet Sauvignon, Cabernet Franc and IAC 138-22 Máximo while using IAC 766 rootstocks has higher anthocyanin content. BRS Violeta grapes show a higher concentration of these pigments when 106-8 Mgt rootstocks are used. Neither the total phenols, nor the antioxidant activity, were influenced by the rootstocks, especially in BRS Violeta and IAC 138-22 Máximo grapes, which presented the highest contents of these compounds using both studied rootstocks. Among white grapes, however, Sauvignon Blanc, IAC 116-31 Rainha and IAC 21-14 Madalena grapes farmed over 106-8 Mgt rootstocks presented higher total phenol levels. Despite being white, hybrid grapes in fruits from IAC 116-31 Rainha, Madalena and IAC 21-14 cultivars, these compounds content, as well as the antioxidant activity, were superior to those found in red grapes from Isabel cultivars.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Isolation and identification of *Talaromyces purpurogenus* and preliminary studies on its pigment production potentials in solid state cultures

Christiana N. Ogbonna<sup>1\*</sup>, Hideki Aoyagi<sup>2</sup> and James C. Ogbonna<sup>3</sup>

<sup>1</sup>Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria.

<sup>2</sup>Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan.

<sup>3</sup>Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

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Pigments from various sources such as annatto seeds, cochineal, beet root, and microalgae are widely used in food, pharmaceutical, cosmetics, textile and other industries. However, these sources of pigments have various limitations such as toxicity and environmental pollution of synthetic pigments, and low productivity of pigments from higher organisms due to long period of growth. The objective of this study was to screen for pigment producing fungi to overcome some of the above limitations. A pigment producing fungus was isolated from soil sample collected from cassava processing site and was identified as *Talaromyces purpurogenus* based on the colony morphology and characteristics, microscopic observation of the conidia and conidiophores and analysis of the gene sequence of internal transcribed spacer (ITS) region of the rDNA. The nucleotide sequence was deposited in Genbank (DDBJ/ EMBL) and was assigned the accession number LC128689. Pigment production by the isolate in solid state cultures using PDA as substrate in Petri dishes was investigated. The optima culture conditions were pour plating method with agar overlay (4 mm thick) and sealed edges, inoculum spore concentration of  $2 \times 10^8$  spores/Petri dish and incubation in dark at 30°C. Under these culture conditions, the red, orange and yellow pigments produced were 11.2, 7.3 and 8.21 unit optical densities per gram of wet agar respectively after 96 h of cultivation. The isolate has good potential for production of different shades of pigments for various applications.

**Key words:** *Talaromyces purpurogenus*, pigments production, solid state cultures, pigment-producing fungi.

## INTRODUCTION

Pigments are very useful compounds with versatile applications. They can be applied in many areas of human life because of their health benefits, aesthetic and

other beneficial values. Pigments find applications in food and feeds (Gupta et al., 2007; Mapari et al., 2010; Manimala et al., 2014), pharmaceuticals, cosmetics,

\*Corresponding author. E-mail: [christiana.ogbonna@unn.edu.ng](mailto:christiana.ogbonna@unn.edu.ng). Tel: +234-8037410250.

wineries and textile (Sharma et al., 2012), for colouring woods for aesthetic values (Robinson et al., 2012), for dyeing of tanned leather (Velmurugan et al., 2009) and for dyeing textile materials (Poorniamal et al., 2013)

Although many synthetic colourants are widely used in industries because they are relatively cheap, some of them can have some detrimental effects on human health and some of them are carcinogenic. Thus a lot of attention is now focused on natural pigments because many of them are known to play some beneficial roles in human health. Some of them have antioxidant (Manimala et al., 2014; Cassia et al., 2005), antimicrobial (Vendruscolo et al., 2014) and anticarcinogenic properties (Deshmukh et al., 2009). These biopigments can be obtained from various natural sources such as higher plants like elderberries (*Sambucus nigra*) (Szaloki-Dorko et al., 2015), black grape skin, red beetroots (*Beta vulgaris*), oil palm fruits, seeds from annatto (*Bixa orellana*), paprika (*Capsicum annum* L.) and tomato (*Lycopersicon esculentum*). Pigments can also be obtained from animals such as insects like kermes (*Kermes vermilio*) and cochineal (*Dactylopius coccus*) (Yilmaz et al., 2014). However, production of pigments from these higher organisms is limited by their low growth rates. Growing of plants to maturity takes at least some months and some acres of lands are required for a large scale production. Pigment production from insects such as *Dactylopius coccus* also takes time and a large number of them are required to extract the pigments for large scale production (Nejad and Nejad, 2013). For example, it was estimated that 155,000 insects are required to produce 1 kg of cochineal dye from *D. coccus* ([https://www.dirtdoctor.com/garden/Cochneal\\_vq1276.htm](https://www.dirtdoctor.com/garden/Cochneal_vq1276.htm); Nejad and Nejad, 2013)

Other natural sources of pigments are microorganisms such as fungi, microalgae, bacteria and lichen. Researches on microbial pigments have been expanding since the past few decades because pigments from these lower organisms are more reliable than those from the higher plants and animals (Dufosse et al., 2014; Chen et al., 2015; Abdel Ghany, 2015; Vendruscolo et al., 2015). Due to the high growth rate of microorganisms, their pigment productivities are high, and not affected by time of the year and seasons while their production has little or no negative environmental impact (Duran et al., 2002). Among the microorganisms employed in the production of pigments, filamentous fungi have been reported to produce large quantities and varieties of pigments because of their ability to synthesize various enzymes that enable them utilize various substrates and catalyze the synthesis of various compounds. Among the fungal kingdom, filamentous fungi of the class ascomycetes are the most popular group known to produce soluble pigments (Dufosse et al., 2014). The first fungus documented to be used for the production of food grade pigments was *Monascus* species (Tieghem, 1884) and since then many strains of *Monascus* have been isolated,

identified and used for pigment production. Some examples include *Monascus purpureus* strain FTC 5391 (Musaalbakri 2006), *Monascus ruber* (Buhler et al., 2015), and *Monascus* sp. strain M9 (Wang et al., 2015). In addition to *Monascus* species, several other species of filamentous fungi such as *Penicillium aculeatum* ATCC 10409 (Afshari et al., 2015), *Penicillium* sp. DLR-7 (Chintapenta et al., 2014) and *Paecilomyces* sp (Cho et al., 2002) have been used for pigment production.

Although many strains of fungi have been isolated and investigated for pigment production, most of the isolates have one or more of the following limitations: Unattractive colour of the pigments, low productivity, low stability of the pigment at high temperature, extreme pH etc., and co-production of some toxins (Chen et al., 2015). There is therefore a need to screen for, isolate and characterize more strains of fungi for pigment production. The aim of this work is to screen for, and isolate pigment-producing filamentous fungi, and to evaluate the ability to produce different shades of pigments on PDA under various culture conditions. In the present report, the authors described the isolation, molecular identification and investigation of pigment production potentials of *Talaromyces purpurogenus* isolated from soil sample collected from cassava processing site using solid state cultures.

## MATERIALS AND METHODS

### Fungi isolation

All the media components used in this study, except otherwise stated, were obtained from Wako Pure Chemical Industries Ltd, Japan. Soil samples were collected from various environments where cassava (*Manihot esculenta* Crantz) tubers are processed into a local staple called 'garri', rice mills, potato farms and corn fields in Eastern part of Nigeria. The soil samples were serially diluted with sterile distilled water and plated out on potato dextrose agar (PDA) in Petri dishes containing 5 µg/ml of chloramphenicol (Pfizer Pharmaceuticals). The plates were incubated at room temperature (25 ±3°C) for seven days. Colonies that showed some pigmentation were picked and sub-cultured in freshly prepared PDA Petri dishes. The sub-culturing was done several times to obtain pure cultures of fungi with high pigment production potential.

### Morphological identification of the fungal isolate

The fungus was inoculated at three points in 9 cm Petri dishes on PDA (Difco) and incubated at 25±3°C for seven days. The isolate was identified by examining the colony morphology, microscopic observation of the hyphae, conidiophores and conidia using light microscope BX51 (Olympus Optical Co., Ltd, Tokyo Japan). Photomicrograph was taken using HK 3.1 CMOS digital camera attached to Olympus BX51 microscope and a scanning electron microscope (LEO Model 1450VP Variable Pressure Scanning Electron Microscope Carl Zeiss, Cambridge, MA, USA).

### DNA isolation, amplification and sequencing

Total genomic DNA was extracted from the isolate using the method

of Manmur (1961). The internal transcribed spacer region (ITS) ITS-5 and 4 were amplified using the method of White et al. (1990). The primers used were Prime STAR HS DNA polymerase (TakaraBio, Japan). The amplified PCR product was sequenced using BigDye Terminator v 3.1 cycle sequence kit (Applied Biosystems, CA, USA) while the sequencing was done using ABI PRISM 3130 X 1 Genetic Analyzer System (Applied Biosystems, CA, USA). The sequence was compared with reference ITS sequence from GenBank at DDBJ/EMBL, using Apron DB-FU 6.0 (Technosuruga Laboratory Co Ltd, Japan). The nucleotide sequence was deposited in Genbank (DDBJ/EMBL) and was assigned the accession number LC128689. Phylogenetic relationships were analyzed by molecular evolutionary genetic analysis using Apron 2.0 software (Technosuruga Laboratory Co Ltd, Japan). A neighbour-joining tree was constructed using the ITS-5.8S rDNA. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade.

#### Sub-culturing and storage of the isolate

The cells were sub-cultured in PDA (39 g/L) test tube slants, incubated at 25°C for seven days and stored in a refrigerator at 4°C. The sub-culturing was done once every six weeks.

#### Pigment production potentials of the newly identified *T. purpurogenus* isolate

This was investigated in solid state cultures using PDA in Petri dishes. Each experiment was performed three times and the average values plus/minus the standard error of the means were plotted. Effects of the following parameters on pigment production were investigated namely: Plating methods (surface plating, pour plating, pour plating with agar overlay, thickness of agar overlay), spore inoculum concentration, illumination and temperature.

#### Effect of plating methods on pigment production by *T. purpurogenus* isolate

PDA (39 g/L) was dispensed at 25 ml per Petri dish after autoclaving. The active *T. purpurogenus* spore suspension (0.1 ml) containing  $1.5 \times 10^7$  spores/ml was used to inoculate the surface of the agar plates and spread out using a glass spreader. In the case of pour plating, the autoclaved PDA (25 mL) in test tubes were left to cool to about 45°C and each test tube was inoculated with 1 ml of  $1.5 \times 10^6$  spore suspension, vortexed briefly to mix and quickly poured into sterile Petri dishes. The plates were prepared in triplicates. After solidification, one set was overlaid with (10 ml) of PDA medium and the edge was taped round with paper tape. The other set was taped round with paper tape without any agar overlay. The three sets of differently treated plates were incubated at 25°C for 96 h. Effects of thickness of the agar layer was also investigated by pouring 10 mL or 15 mL of agar on top of solidified inoculated PDA, resulting respectively to 2.66 and 4.00 mm thick un-inoculated PDA on top of seeded PDA in Petri dishes. All other culture conditions were as described before.

#### Effect of spore inoculum concentration on pigment production by *T. purpurogenus* isolate in solid state culture

PDA at 39 g/L was prepared and dispensed in 25 ml aliquots into test tubes as described before. The test tubes were sterilized by autoclaving at 121°C for 15 min. After cooling to 45°C each test tube was then inoculated with one of the following spore inoculum concentrations  $2 \times 10^6$ ,  $2 \times 10^7$ ,  $2 \times 10^8$  or  $2 \times 10^9$  per test tube. Each

test tube was prepared in triplicates and poured into Petri dishes as described before. The surface of each Petri dish was overlaid with 15 ml of sterile PDA and taped round with paper tape. The Petri dishes were incubated at 25°C for 96 h.

#### Effect of light illumination on pigment production by *T. purpurogenus* isolate

Pour plate with 15 ml of sterile PDA over laid on top and sealed round with paper tape were prepared in triplicates and one set was incubated in the dark and the other under white unscreened illumination using white fluorescent lamp. The light intensity was measured with lux meter (Yokogawa digital lux meter 51011 series Japan). The inoculum spore concentration was  $2 \times 10^8$  spores per Petri-dish, the incubation time and temperature were 96 h and 25°C, respectively while the light intensity was  $50 \mu\text{mol}/\text{m}^2 \cdot \text{s}$  ( $1 \text{ lux} = 0.0185 \mu\text{mol}/\text{m}^2 \cdot \text{s}$ ).

#### Effect of temperature on pigment production by *T. purpurogenus* isolate

Pour plates with seal were prepared in triplicates as described previously. One set was incubated at 25°C and the other at 30°C. The inoculum spore concentration was  $2 \times 10^8$  spores per Petri-dish while incubation was carried out in the dark for 96 h.

#### Pigment extraction for spectrophotometric determination of the unit optical density

At the end of each cultivation, a disk of the PDA (about one gram) was cut from three points in a Petri dish using a sterile cork borer. Each disk was accurately weighed and placed in 10 ml of either water or methanol in a test tube. The pigment was left to extract overnight with shaking at 100 rpm at room temperature  $25 \pm 3^\circ\text{C}$ . After that, the extract from each disk was filtered and the filtrate was used for measurement of the pigment unit optical density per gram of agar (UOD/g). Extract from un-inoculated PDA was used as a blank.

#### Spectrophotometric determination of pigment optical density

The yellow, orange and red pigments were measured at wave lengths of 400, 460 and 500 nm respectively using UV visible spectrophotometer (Shimadzu Model UV-1200) according to the method of Cho et al. (2002). Filtrate from sterile PDA was used as a blank.

#### Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) and where significant differences were observed, the means were separated by the Least Significant Difference (LSD) ( $P = 0.05$ ) test.

## RESULTS

### Morphological characteristics of the isolate on PDA culture

The surface view (obverse) and the bottom view (reverse) of the isolate after cultivation on PDA for 7 days



**Figure 1.** PDA plates showing the colonies of the isolate. The isolate was cultivated in PDA at 25°C for 7 days. A: Surface (obverse); B: Bottom (reverse) views. C: Micrograph of the isolate, showing the conidiophores and conidia.

at 25°C are shown in Figure 1A and B, respectively. The fungus produced whitish colonies on PDA plates within the first three days of incubation at 25°C but turned light brown with increase in incubation period. As revealed by the electron micrograph of the reproductive morphology of the newly isolated *T. purpurogenus* (Figure 1C), the conidiophores bear numerous ellipsoidal conidia on the secondary phialides.

#### Molecular identification of the isolate

Based on the Apron DB-FU 6.0 BLAST, the relationship of the ITS-5.8S rDNA base sequence of the isolate with some related fungal species in the data base is shown in Table 1. All the *Talaromyces* strains in the database showed more than 98.8% base sequence similarity to the new isolate. The neighbour-joining tree of the ITS-5.8S rDNA region showing phylogenetic placement of the isolate is shown in Figure 2 where T denotes Ex-type strain while NT denotes Ex-neotype strain while numbers at the branching nodes represent bootstrap values. On

the basis of the morphological characteristics and base sequence similarity, the isolate was identified as *T. purpurogenus* (Stoll) (Samson et al., 2011).

#### Effect of plating method on pigment production by *T. purpurogenus* isolate

The results of three plating methods namely: Surface plating, pour plating without and with agar layer on the surfaces are shown in Figure 3A and B. Pour plating with agar layer on top produced the highest pigment (UOD) among the three plating methods examined. Using the pour plating with agar overlay method, the amount of red pigment (1.73 UOD/g) was significantly higher than the orange (1.05 UOD/g) and yellow (1.03 UOD/g) pigments ( $P < 0.05$ ) but there was no significant difference between the amounts of orange and yellow pigments ( $P > 0.05$ ). Under pour plating without agar overlay, the red, orange and yellow pigments were 0.36, 0.29 and 0.39 UOD/g of agar respectively. Surface plating produced the least UOD of the three pigments viz: 0.15, 0.11 and 0.18 of



**Table 1.** Comparison of the ITS-5.8S rDNA Base Sequence of the isolate with some related fungal species in the database.

Strain	Code	Accession No.	Sequence similarity (%)
<i>Talaromyces flavus</i>	XSD-46	EU273527	562/564 (99.6)
<i>T. flavus</i>	SP5	JF509738	559/562 (99.5)
<i>T. flavus</i>	wb239	AF455513	560/564 (99.3)
<i>T. flavus</i>	wb252	AF455509	559/562 (99.3)
<i>T. Purpurogenus</i>	CBS108923	JX965236	561/563 (99.6)
<i>T. purpurogenus</i>	CBS113158	JX965235	561/563 (99.6)
<i>T. purpurogenus</i>	CBS113161	JX965234	561/563 (99.6)
<i>T. purpurogenus</i>	DTO193H5	JX965233	561/563 (99.6)
<i>T. purpurogenus</i>	DTO189B4	JX965231	561/563 (99.6)
<i>T. funiculosus</i>	UOA/HCPF<GRC>:13814	KC254067	560/562 (99.6)
<i>T. purpurogenus</i>	DTO173E6	JX965230	560/563 (99.5)
<i>T. purpurogenus</i>	DTO189A1	JX315661	541/543 (99.6)
<i>T. purpurogenus</i>	CBS122434	JX315663	541/543 (99.6)
<i>T. funiculosus</i>	NG_p14	HQ115695	541/543 (99.6)
<i>T. purpurogenus</i>	DTO193H1	JX965232	538/540 (99.6)
<i>T. purpurogenus</i>	CBS184.27	JX315665	540/543 (99.4)
<i>T. purpurogenus</i>	CBS286.36T	JN899372	556/563 (98.8)
<i>Penicillium</i> sp.	WD02	KC678995	526/531 (99.1)

red, orange and yellow, respectively. Statistically, there was no significant differences in the UOD of all the pigments produced under both pour plating without agar layer and surface plating ( $P > 0.05$ ).

#### Effect of pour-plating with agar layer on sporulation and pigment production under sealed condition

The results of these two plating methods are shown in Figure 4. Pour plating, with agar overlay and sealed edges produced significantly higher UOD/g of the three pigments (red, orange and yellow) than plates without agar overlay with sealed edges ( $P < 0.05$ ). The average pigment UOD/g were 1.13, 0.71 and 0.64 UOD/g agar for red, orange and yellow pigments respectively in plates with agar overlay and sealed edges. Furthermore, the red pigment UOD was significantly higher than those of orange and yellow ( $P < 0.05$ ) under this plating method. Plates without agar overlay but with sealed edges produced 0.33, 0.26 and 0.34 UOD/g which were about 3.38, 2.75 and 2.00 times respectively, lower than the values obtained under pour plates with agar layer and sealed edges. In the case of sealed plates without agar overlay, there was no significant difference in the UOD of red, orange and yellow pigments.

#### Effect of the thickness of the agar overlaid on top of pour plates on pigment production

The results of the effects of thickness of agar layer (4.00 and 2.66 mm) on pigment production are shown in Figure

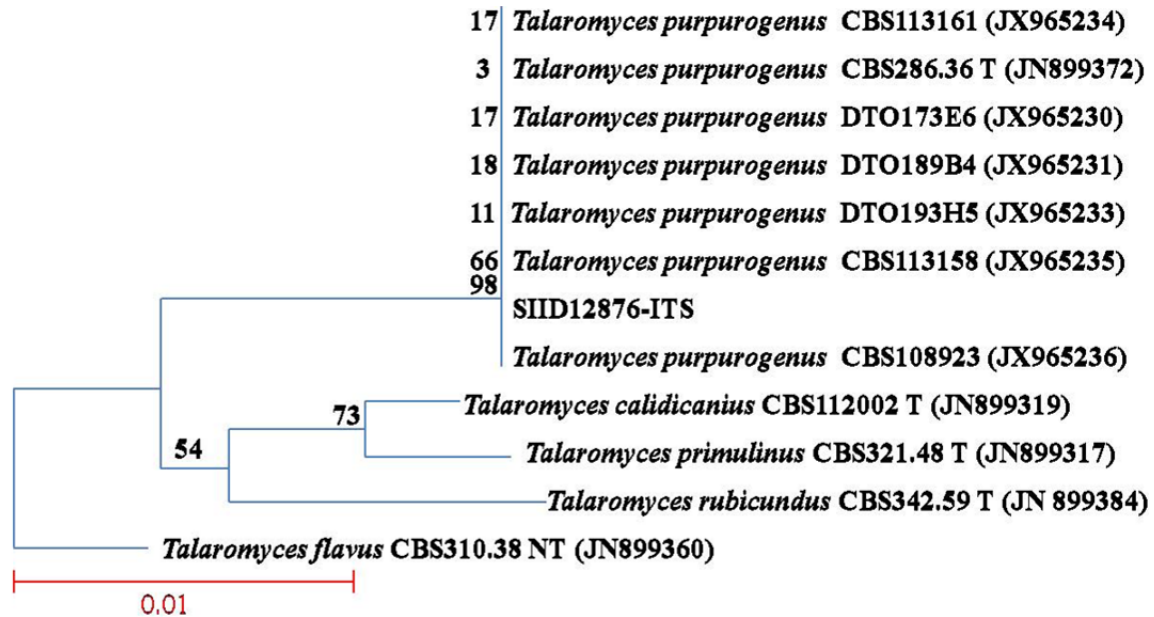
5. Thick agar (4.00 mm thick) overlay produced significantly higher UOD of red, orange and yellow pigments than using thin (2.66 mm) agar layer ( $P < 0.05$ ). With thick agar layer, 1.45 UOD/g agar of red pigment was produced against 0.40 UOD/g produced when thin agar layer was used. For orange and yellow pigments, 0.95 and 0.96 respectively were produced by using thick agar layer against 0.30 and 0.38 produced when thin agar layer was used.

#### Effect of spore inoculum concentration on pigment production

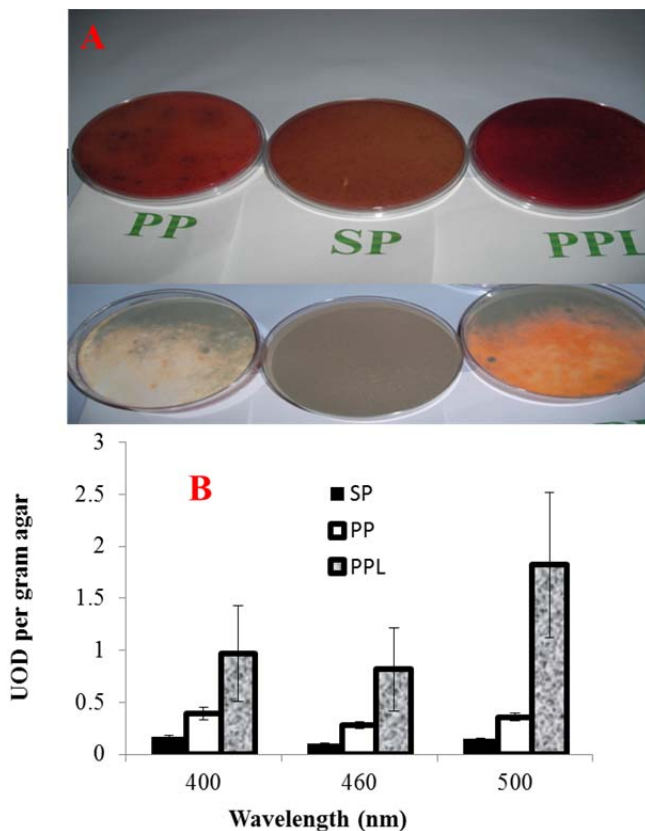
When the optimum plating method of pour plating with agar overlay and sealed edges was confirmed to be the best, the optimum spore inoculum concentration was investigated. The results of the effects of spore inoculum concentration per Petri dish on pigment production are shown in Figure 6. The pigment UOD increased with increase in inoculum concentration up to a certain level. Spore inoculum concentration of  $2 \times 10^8$  spores/Petri dish was the optimum for production of the red, yellow and orange pigments. The amount of pigments produced with spore inoculum concentration of  $2 \times 10^8$  spore/plate was significantly higher than those produced with other spore inoculum concentrations tested ( $p < 0.05$ ).

#### Effect of illumination on pigment production

The results of the effects of light illumination on pigment



**Figure 2.** Neighbour-joining tree of the ITS-5.8S rDNA region showing phylogenetic placement of the isolate. T denotes Ex-type strain while NT denotes Ex-neotype strain. Numbers



**Figure 3.** Effect of plating method on pigment production by *T. purpurogenus*. SP, Surface plating; PP, pour plate method; PPL, pour-plate with agar layer. (A) Top and Bottom views of pigments in agar plates after 96 h of cultivation. (B) Data presented are the means  $\pm$  SE (n = 3). Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively.

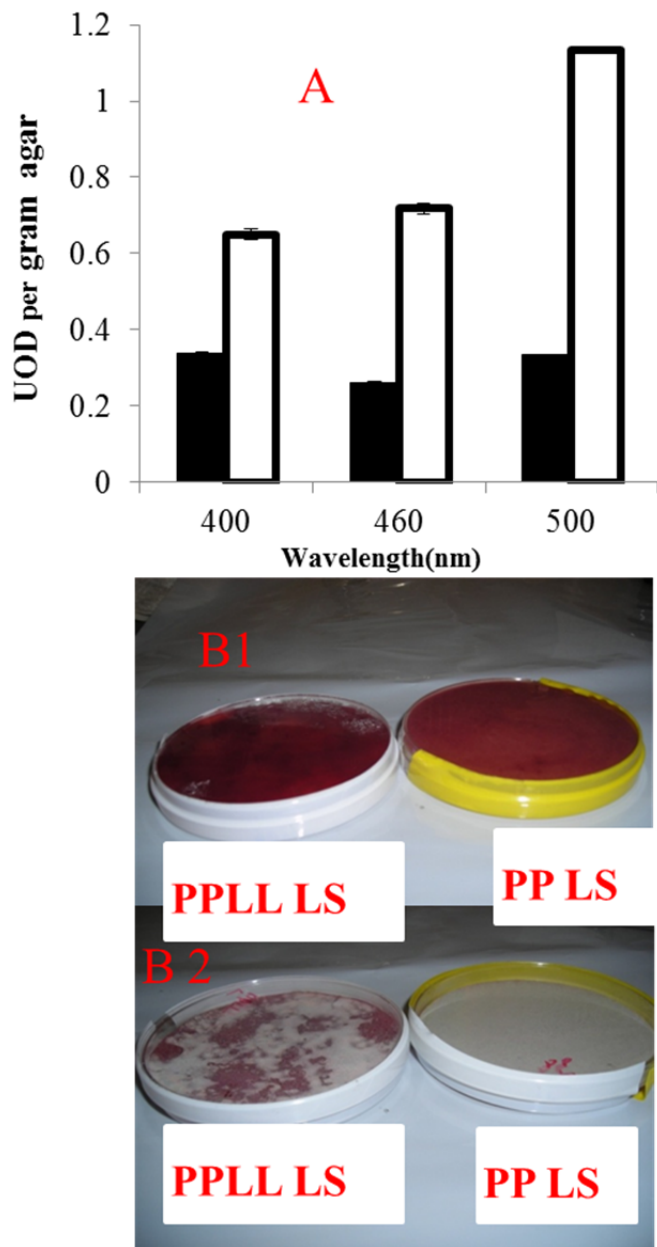
production are shown in Figure 7. Production of red, orange and yellow pigments were similar when grown in the dark and under light conditions. The UOD of red pigments were 5.83 and 5.64 UOD/g under dark and light conditions respectively. Also the UOD of orange and yellow pigments were 4.12 and 4.74; and 3.91 and 4.63 UOD/g under dark and light conditions respectively. On the whole, light illumination had no significant effect on the production of the three types of pigments ( $P > 0.05$ ).

#### Effect of temperature on pigment production

The results obtained after cultivating at 25 and 30°C are shown in Figure 8. Pigment production was better at 30°C than at 25°C. The average pigment concentrations (UOD/g) obtained at 30°C were 11.20, 7.30 and 8.21 for red, orange and yellow pigments respectively. At 25°C the average values obtained were (UOD/g) 4.75, 3.02 and 3.28 for red, orange and yellow pigments respectively. These were about 2.35, 2.41 and 2.50 times lower than what was produced at 30°C for red, orange and yellow pigments respectively. Pigment production was significantly reduced at 35°C (data not shown).

#### DISCUSSION

Species of *Talaromyces* have been reported to produce various pigments both in suspended and solid state cultures (Frisvad et al., 2013; General et al., 2014). The present study has also shown that a strain of *T. purpurogenus*, isolated from soil sample collected from



**Figure 4A.** Effect of pour-plate method with agar layer on sporulation and pigment production under sealed condition. PPLL LS, Pour plating with agar layer (white bar); PP LS, Pour-pating without agar layer (black bar). Figure 4B 1 and 2 are bottom and surface views of pigments in agar plates with the two plating methods after 96 h of cultivation. Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively. Data presented are the means  $\pm$  SE (n = 3).

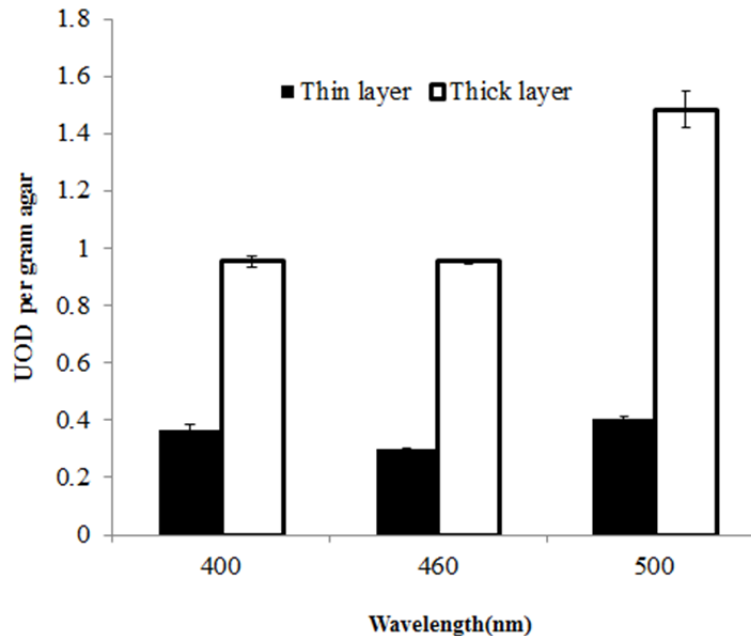
cassava processing site is capable of producing diffusible red, orange and yellow pigments in agar cultures under various conditions. These pigments have potential applications as colouring agents in textile, cosmetics, pharmaceutical, food, and wood industries. The advantages of using solid state culture in pigment

production include the possibility of using cheap substrates, lower cost of purification since contamination by media components is minimal, and also generation of less effluent (Babitha et al., 2006; Kapilan, 2015).

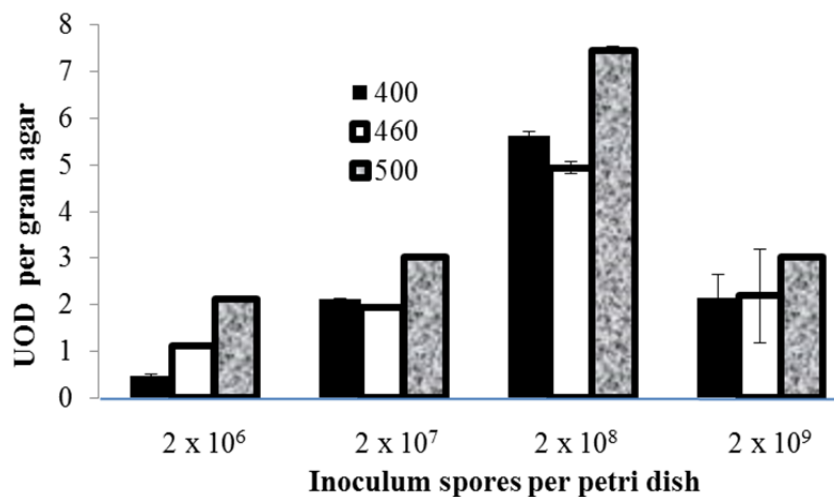
Plating method has a profound effect on metabolite production by fungi. With surface plating, there was profuse hyphal growth and sporulation with less pigment. This might be because of high aeration and evaporation rate in Petri dishes. Pour plating method is an effective method of cultivating fungi for metabolite production and it is interesting to note that production of all the three pigments was higher in pour-plate method than in surface plating. With pour plating, the spores were more evenly distributed on the agar plates which reduced competition for nutrients. It is also very interesting to observe that pour plating with an agar overlay and sealed edges produced the highest quantity of pigments among surface plating and pour plating without agar overlay. Overlaying the agar surface inside Petri dish with agar insulated the organism, conserved the moisture contents, reduced aeration and thus prevented profuse vegetative growth thereby channelling all the energy to pigment production. Sealing the Petri dishes with paper tape also provided additional insulation and prevented much water loss by evaporation from the culture. This seems to be the first report on using this technique of pour plating with agar overlay for pigment production by a fungus. This knowledge will be useful in controlling the moisture content/humidity when grains and other solid substrates are used for large scale metabolite production.

Spore inoculum concentration is an important parameter in any fermentation process because too high spore inoculum will deplete the nutrient for vegetative growth soon after inoculation. Under this condition, there will be little or no nutrient for metabolite production. On the other hand, too little cell inoculum will result in sluggish growth and inefficient nutrient utilization. Velmurugan et al. (2010a) also reported that spore inoculum concentration of 4 ml containing  $6 \times 10^5$  spores/ml was the best for inoculation of 5 g of substrate for solid state fermentation. General et al. (2014) reported optimum inoculum volume of  $1.8 \times 10^6$  spores per gram solid substrate during pigment production by *Talaromyces amestolkiae* using macroalgal biomass as the substrate.

Fungi, respond to light during growth and metabolite production just like most prokaryotes and eukaryotes. Our results showed that pigment production was favoured more by incubating in a dark place than under light. The effects of light on pigment production by fungi have been studied by many researchers. Buhler et al. (2015) reported that during cultivation of *M. ruber*, growth and pigment production were inhibited in Petri dishes and baffled flasks exposed to direct illumination. Velmurugan et al. (2010b) also noted that growth and pigment production by *M. purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides* and *P. purpurogenum* were higher under dark condition than when exposed to



**Figure 5.** Effect of the thickness of agar overlay on pigment production. The thickness of the thin layer was 2.66 mm while that of the thick layer was 4.0 mm. This was done by pouring 10 ml and 15ml of un-inoculated PDA respectively onto seeded solidified potato dextrose agar in Petri dishes. Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively. Data presented are the means  $\pm$  SE (n = 3).

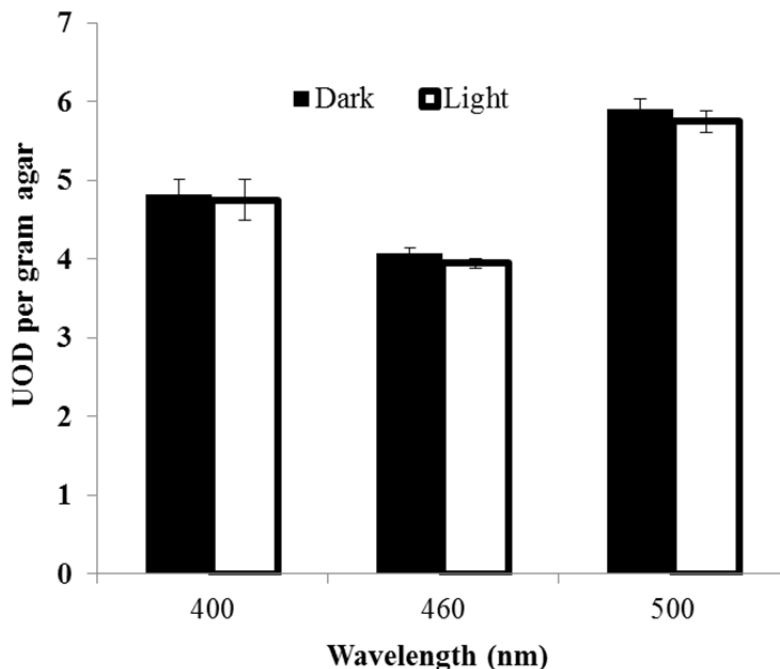


**Figure 6.** Effect of spore inoculum concentration on pigment production. Three levels of spore concentration were used to inoculate 25 ml of autoclaved PDA in test tubes. The inoculated PDA was then poured into Petri dishes, overlaid with 15 ml of agar after solidification, taped round and incubated at 25°C for 96 h. Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively. Data presented are the means of  $\pm$  SE (n = 3).

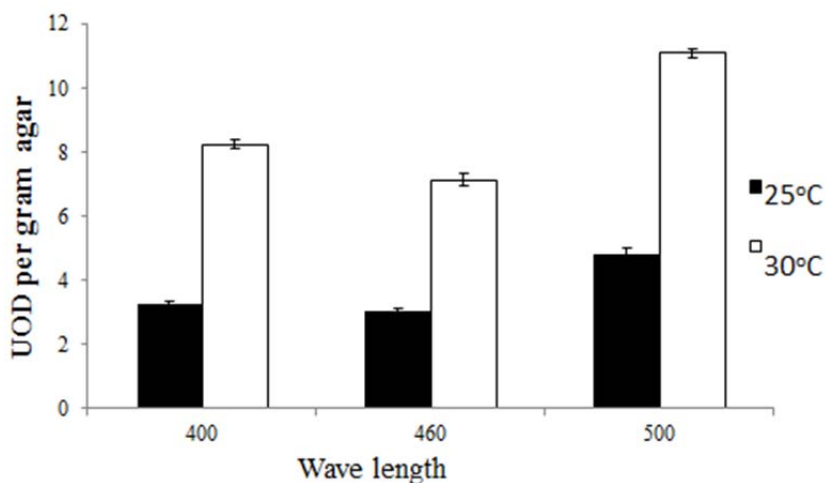
lights of various wavelengths. In the case of *M. purpureus*, Velmurugan et al. (2009) reported that incubation in total darkness increased red pigment production but illumination resulted in total suppression of

pigment production.

Most fungi are mesophiles whose growth and metabolite production occur mostly within the temperature range of 25 and 30°C. The optimum



**Figure 7.** Effect of illumination on pigment production. PDA plates were prepared in triplicates by pour plating. The plates were inoculated with  $2 \times 10^8$  spores/ mL taped round. One set was incubated in dark and the other under illumination with light intensity of 150 micro mol/m<sup>2</sup>s. Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively. Data presented are the means of  $\pm$  SE (n = 3).



**Figure 8.** Effect of temperature on pigment production by *T. purpurogenus*. Pour plates with agar overlay and seal were prepared in triplicates. One set was incubated at 30°C and the other set was incubated at 25°C. The inoculum concentration was  $2 \times 10^8$  spores per Petri-dish while incubation was in dark. Wavelengths of 400, 460 and 500 nm denotes yellow, orange and red pigments, respectively.

temperature for pigment production in solid state culture in Petri dish by this isolate of *T. purpurogenus* was 30°C. This was in agreement with the results of pigment

production by *Penicillium aculeatum* ATCC 10409 (Afshari et al., 2015), *M. ruber* (Said et al., 2010) and *M. purpureus* CMU001 (Nimnoi and Lumyong, 2011). With

all these species, the optimum temperature was reported to be 30°C. When the newly isolated *T. purpurogenus* was cultivated at 35°C there was good growth but pigment production was very low.

Under the present experimental conditions, red, orange and yellow pigments of 11.2, 7.28 and 8.21 UOD per gram of wet agar were obtained. This is comparable with the results of other researchers who used dried fermented substrates considering the water content of the substrate in the present study. For example, Velmurugan et al. (2009) reported 30.8 and 25.5 absorbance unit per gram of dry fermented substrate for red and yellow pigments respectively using jackfruit seed supplemented with monosodium glutamate as substrate. Pigment production by the newly isolated *T. purpurogenus* in suspended liquid culture under various culture conditions is currently under investigation. On the whole, the above results have shown that pigment production by *T. purpurogenus* is highly affected by temperature, inoculum concentrations and plating methods.

## Conclusion

A pigment producing *T. purpurogenus* was successfully isolated from the soil and identified based on the macroscopic and microscopic morphology and the ITS-5.8S rDNA base sequence. All the *Talaromyces* strains in the database showed more than 98.8% base sequence similarity to our new isolate. The optimum plating method for pigment production by this isolate was pour plating with thick agar layer of 4 mm on top and sealed edges. The optimum spore inoculum concentration was  $2 \times 10^8$  per Petri dish. Pigment production was not affected by illumination conditions. The unit optical density of pigments produced at 30°C was higher than the values obtained at 25°C. The results of this study have revealed that the new isolate of *T. purpurogenus* has high potentials for industrial pigment production.

## CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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