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Full Length Research Paper

Energy balance of elephant grass biomass for power generation by direct biomass combustion

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In Brazil, elephant grass has been used for thermal energy production, due to the high productivity, energy efficiency, biological nitrogen fixation (BNF) and biomass quality for energy generation by direct burning. The use of biomass depends mainly on the energy balance and biomass characteristics for energy production. The aim of this study was to evaluate the biomass of dry matter yield, qualitative biomass variables and energy balance of two elephant grass genotypes. The experimental design was completely randomized blocks with two elephant grass cultivars (Gramafante and Roxo) and four replications per treatment. Five cultivation cycles were studied. Biomass yield and the contents of acid detergent fiber, lignin, cellulose, ash and calorific value were evaluated. The biomass yields of elephant grass cultivars varied between 12 and 24 Mg ha^{-1}, where the lowest yields were presented by the cultivar Roxo, and the higher yields by Gramafante. The contents of fiber, lignin, cellulose and calorific value did not vary among the genotypes studied. An energy balance was calculated for the complete biomass production lifecycle of elephant grass and the overall energy output/input ratio was 15.1:1. The results show that the elephant grass has highly suitable materials for the production of energy by direct burning.

Key words: *Pennisetum purpureum,* agroenergy, dry matter.

INTRODUCTION

In recent decades, the search for alternative sources of energy in lieu of the use of fossil fuels has been growing globally. Renewable energy sources such as biofuels and biomass are an important strategy to reduce fossil fuel use, especially for countries with large areas available for agriculture and satisfactory rainfall distribution, such as Brazil (Morais et al., 2013). Apart from the success in mitigating greenhouse gases (GHGs) by the Brazilian ethanol program (Boddey et al., 2008; Macedo et al., 2008), there is a growing interest for plant biomass to supply the sectors of the economy in rural areas depending on heat generation for drying processes and

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for electricity generation (Morais et al., 2013).

In the world, the biomass of Poaceaes family of C4metabolism with high productive potential such as *Miscanthus* spp., *Panicum* spp. and *Saccharum* spp.have been highlighted in this scenario (O'Loughlin et al., 2017; Fei et al., 2017). C4 photosynthesis of plants boosts productivity in some of the planet's most ecologically and agronomically important species (Huang et al., 2017). In Brazil, some studies have been carried out with elephant grass (*Pennisetum purpureum*) (Basso et al., 2014; Morais et al., 2013; Quesada, 2005; Samson et al., 2005). Morais et al. (2009) found some ascending genotypes for biomass production for bioenergy generation purposes and observed lignin, cellulose and ash contents suitable for use in direct burning. Morais et al. (2013) quantified greenhouse gas emissions in the elephant grass biomass production system derived from soil preparation and nitrogen fertilization and took an important step to characterize the elephant grass inclusion scenario in the energy scenario.

The main ways in which energetic use of elephant grass biomass has been studied, are the use in thermoelectric plants and for direct combustion in ceramic industries to replace wood and coal. Elephant grass biomass is cut in the field, dried in an appropriate place, and then transported for direct burning. Apart from high dry matter production, the plant material should suit quality parameters for reaching the optimal energetic efficiency, which means high levels of fiber and lignin and low levels of water, N and ash (Lemus et al., 2002; McKendry, 2002). Quesada (2005) and Morais et al. (2013) quantified dry matter production of 30 Mg ha^{-1} $year^{-1}$ with fiber and lignin contents above 60 and 10% respectively, as well as low protein and ash content, using only P and K fertilization, and two cuts per year.

For elephant grass growing in poor N soils, the average dry matter production after two cuts per year was about 30 Mg ha^{-1}, and fiber and lignin contents matched the desirable parameters for energy production from direct burning (Quesada, 2005). Protein content was remarkably reduced in comparison with common levels observed in elephant grass genotypes, destined for forage production. Biomass yield levels were not drastically reduced, in comparison with the ones in fertilized systems (Quesada, 2005). The suitability of the biomass for energy production, together with the possibility of two cuts per year, increase the potential of use of this crop for energy production purposes (Samson et al., 2005).

Another factor that influences the sustainability of the use of biomass is its energy balance. Samson et al. (2005) found that for the agricultural stage of elephant grass production, a value of 21:1 was obtained, showing that for each unit of fossil energy used in the elephant grass production process, 21 units of renewable energy were obtained. This condition is valid when the material is used directly for combustion. For any procedure that promotes changes in the state of biomass, for example, transforming the biomass into coal, there will be changes in the energy balance. Considering a yield of 30 Mg ha^{-1} of dry matter (DM), the crude energy produced is 493 GJ ha^{-1} $year^{-1}$ (Samson et al., 2005).

As input quantities and biomass yields vary according to cropping site and investments made during the process, it is of utmost importance that the necessary measurements are made in each region of the country for a more real characterization. Therefore, the objective in this study was to quantify the biomass yield, qualitative characteristics of the biomass and the energy balance of two elephant grass genotypes, in five cycles, aiming to contribute to optimizing their use for energy generation by direct burning.

MATERIALS AND METHODS

Location and experimental description

This study was performed at the experimental area of the Federal Institute of Roraima- Campus Amajari, located in the town Amajari of Roraima. The study period was between March 2014 and September 2016. The precipitation and minimum, medium and maximum temperatures corresponding to the studied cycles period are shown in Figure 1. The experimental design was completely randomized blocks with four treatments and four replications. The soil of the region was classified as Yellow Argisol (Ultisol), presenting the 0.62 mg dm^3 of phosphorus and 0.03 cmolc dm^3 of potassium availability, besides the 0.14 and 0.06 comlc dm^3 of calcium and magnesium respectively, observed in the soil analysis. The plots consisted of 5 lines with 5 m length and space of 1 m, totalizing an area of 25 m^2 per plot.

Climate variables

The results of precipitation and minimum, medium and maximum temperature are shown in Figure 1. In the three years of study, the highest precipitations were observed between April and August. In this interval, precipitations observed surpassed 300 mm per month. In the remaining months, the rains rarely exceeded 40 mm. As for temperatures, on average, they were close to 30°C in the five cycles studied and did not influence the development of elephant grass genotypes.

Soil preparation and fertilizer application

The area of the experiment remained fallow during the previous 5 years, being only managed to control the spontaneous vegetation by weed wrecking. The stages of soil preparation consisted of a plowing followed by two harrowings performed after fifteen days. After the harrowing, furrows were made to plant elephant grass. In these furrows, single superphosphate fertilizers, potassium chloride and a set of micronutrients in the commercial form of FTE BR12 were applied. The recommendation was based on the soil analysis and corresponded to 80 kg ha^{-1} of P_2O_5, 60 kg ha^{-1} of K_2O and 50 kg ha^{-1} of FTE BR12. After each cut, the nutrients extracted by the crop were replaced.

The doses of P, K and micronutrients were applied based on the mean accumulation of these nutrients in elephant grass plants (Andrade et al., 2005; Moreira et al., 2006).

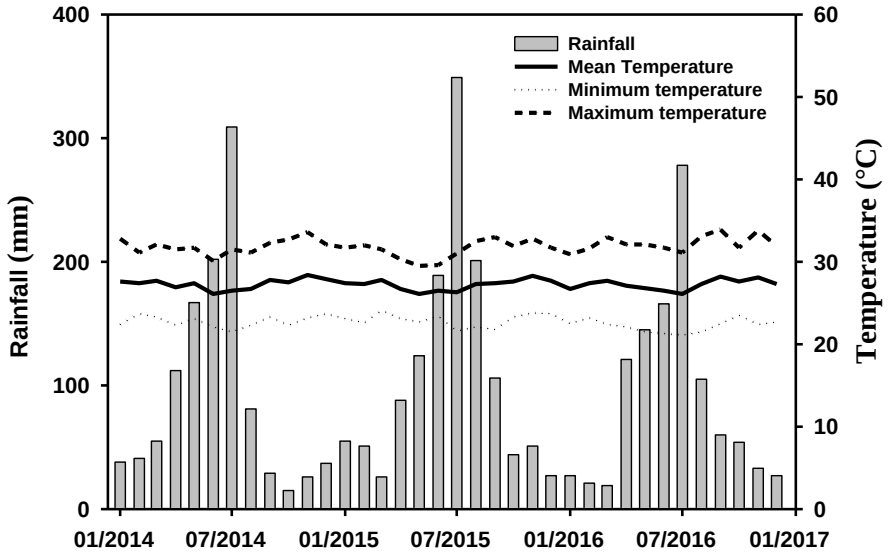

Figure 1. Rainfall precipitation and minimum, medium and maximum temperatures during the experimental period between December 2014 and January 2017, in Boa Vista-RR. Source: Data from INMET (2014 to 2016).

Biomass yield

The treatments were evaluated by harvesting elephant grass biomass after six months of planting. The biomass of the plots was weighed fresh, without separating stem and leaves, and after that, sub-samples were taken, then put in a drying oven at 65°C until weight stabilization and the dry matter fraction of plants was determined. The plant samples were pre-ground in a Wiley type mill (2-mm-sieves) for the chemical analysis performed.

Chemical analysis

The analysis related to neutral detergent fiber (NDF) and its components and ashes were carried out according to the methodology described by Van Soest and Wine (1968). The upper calorific value (UCV) was determined by the calorimeter pump method with the digital calorimeter (model C-200, IKA). The tests were performed according to the ABNT NBR 8633/84 standard and the instrument manual. Those determinations were carried out in partnership with the forage crops department of the Animal Science Institute of the Federal Rural University of Rio de Janeiro.

Energy balance

Each agricultural production activity (tillage, planting, fertilization, irrigation, harvesting, transport and storage) was included in the energy balance. Each input and values used for energy balance were derived by the authors from the field study of Macedo (1997, 1998).

For the energy balance calculations, the average yield of the two elephant grass genotypes in the five crop cycles was considered. The average calorific value data were used to estimate the total energy obtained from elephant grass biomass in an area of 1 hectare. On average, a calorific value of 3822 kcal per kg of elephant grass was observed; it can be considered that it corresponds to 16 GJ/ha of gross energy. The diesel conversion factor of 0.0478 GJ/l and the values for harvesting and maintenance of agricultural machines obtained under laboratory conditions for sugarcane were used. For the transportation of biomass after harvesting and drying, an average distance of 8 km (Round Trip) and 15 min for each stretch were considered.

Statistical analysis

The statistical procedures were those of the SAEG 9.1 (Universidade Federal de Viçosa, 2007). Normality and homogeneity of variance of errors were analyzed using the Lilliefors and Cochran and Bartley tests, respectively. The required conditions were met in all cases. The analysis of variance was performed with the application of the F test. Differences between means were separated by the use of Tukey's test at 5% probability. The climate data were collected on the National Institute of Meteorology of INMET website - Boa Vista Station.

RESULTS AND DISCUSSION

Biomass yield

In the first and fourth cycles, there was statistical difference between genotypes, and dry matter accumulation was always higher for Gramafante cultivar as compared to Roxo, with values of 22.7 and 15.8 of Mg ha^{-1} for Gramafante and 18.5 and 12.3 of Mg ha^{-1} for Roxo.

In the comparison between cycles, for the Gramafante genotype, there was statistical difference between the second and fourth cycles when compared with the other cycles. For the Roxo genotype, the first and the third cycles presented statistical differences when compared with the others cycles (Table 1). The precipitation differences between the first and third cycles as

Table 1. Dry biomass yield of two elephant grass genotypes in a Yellow Latosol in Amajari-RR.

Genotypes	Dry matter yield (Mg per ha^{-1})				
	1° Cycle	2° Cycle	3° Cycle	4° Cycle	5° Cycle
Elephant grass cv. Roxo	18.5^{Ba}	15.3^{Ab}	21.2^{Aa}	12.3^{Bb}	16.6^{Ab}
Elephantgrass cv. Gramafante	22.7^{Aa}	17.7^{Ab}	23.1^{Aa}	15.8^{Ab}	19.2^{Aa}
Mean	20.6	16.5	22.15	14.05	17.9
Accumulated rainfall (mm) *	955	210	1057	189	875

Means followed by the same capital letter in the columns and small letter in the rows do not differ from each other by the Tukey's test at 5%* and 1%** of probability.

Table 2. Acid detergent fiber (ADF), lignin, cellulose (expressed in %) and calorific value (expressed in Kcal/kg) of the Poaceaes studied (3rd cycle).

Treatments	[a]ADF	[a]Lignin	[a]Cellulose	[b]Calorific value
Elephant grass Cultivar Roxo	39.5	7.3	29	3876^{A}
Elephant grass Culivar Gramafante	39.4	6.9	31	3769^{A}
Mean	36.0^{ns}	6.0^{ns}	28.7^{ns}	3664 ^{ns}
CV (%)	11.3	12.2	16.7	12.9

Means followed by the same letter in the column do not differ by the Tukey's test at 5%* and 1%** of probability. ns, Not significant.

compared to the second and fourth was greater than 700 mm precipitation.

Considering the average yield of biomass production in the five cycles for each genotype, it was observed that the genotype Gramafante presented productivity that is 15% higher than the Roxo genotype. In the period of 1 year or 2 harvests, the biomass production of the genotypes studied exceeded 30 Mg ha^{-1}. The high productive potential for biomass production observed in elephant grass in the present study is consistent with those already reported in literature.

Morais et al. (2013) observed dry biomass yield of elephant grass above 30 Mg ha^{-1} in six months of cultivation. Andrade et al. (2005) and Queiroz Filho et al. (2000) observed biomass production of more than 40 Mg ha^{-} year^{-1} of dry matter produced annually. These data corroborate those obtained by Quesada (2005), who found an average yield of 35 Mg ha^{-1} of DM in 15 months of cultivation with the application of 80 kg of N fertilizer, working on a Planosol and with all the genotypes of this study. Queiroz Filho et al. (1998) found productivities of 19 Mg ha^{-1} year^{-1} of MS even with fertilization of 100 kg of N ha^{-1}, in the form of ammonium sulphate, values far below those found in this study, in which, if the time of two yields is summed up (15 months), a yield of up to 45 Mg ha^{-1} of DM is observed.

The obtained data surpass productivities of other species used as source of energy. Schemer et al. (2008) in studies with *Panicum virgatum* obtained ethanol from cellulose in the biomass, based on an average productivity of 8.15 Mg. ha^{-1}. This yield is about 75% lower than that obtained by Quesada (2005), which showed an average of 35 Mg. ha^{-1} of MS in studies with elephant grass. It is also worth noting that in the elephant grass culture, it is possible to achieve an energy balance of up to 21.3 (Samson et al., 2005) and in studies carried out by Schemer et al. (2008) balance reached 5.4.

Lemus et al. (2002) in studies with 20 cultivars of *P. virgatum*, obtained an average of 9 Mg. ha^{-1} of biomass with application of 100 kg of N ha^{-1}, yield that can also be considered far below those obtained with elephant grass by Quesada (2001) and Quesada (2005). Botrel et al. (2000), studying new elephantgrass clones, among them, Cameroon, CNPGL 91 F27-01 and CNPGL 91 F06-03, found annual average productivity of 31, 43 and 37 Mg. ha^{-1} year^{-1} of DM respectively. Danalatos et al. (2007) working with *Miscanthus sinensis* under two doses of N fertilizer (50 and 100 kg of N ha^{-1}), observed that there was no significant response to these treatments, obtaining an average yield of 27 Mg. ha^{-1} of DM in 270 days of cultivation. Quesada (2005) found values of up to 30 Mg. ha^{-1} of DM in eight months of cultivation in Cameroon and Gramafante genotypes in the application of N-fertilizer.

Qualitative parameters of biomass

There was no significant difference between the studied genotypes or the cultivation cycles for the fiber, cellulose, lignin and calorific values (Table 2). The levels of acid detergent fiber were close to 40% in the two genotypes studied. The lignin contents were 6.9 for the genotype Roxo and 7.3 for the Gramafante. Regarding the contents

Table 3. Energy balance of elephant grass biomass production.

Field activities	Equipments and vehicles used	Total consumed energy (GJ/ha/year)
Lime application	Tractor Massey Fergusson 290 consuming 20 L/diesel/ha every 3 years	0.31
Heavy plough	Tractor Agrale Deutz BX 4150 consuming 15 L/diesel/ha every 3 years	0.239
Leveling harrow	Tractor Agrale Deutz BX 4150 consuming 8 L/diesel/ha every 3 years	0.127
Furrowing and fertilizer application	Tractor Agrale Deutz BX 4150 consuming 11.6 L/diesel/ha every 3 years	0.185
Distribution of setts	Tractor Massey Fergusson 290 consuming 4.8 L/diesel/ha every 3 years	0.076
Closing of furrows	Tractor Massey Fergusson 290 consuming 2.5 L/diesel/ha every 3 years	0.039
Harvest	Tractor Agrale Deutz BX 4150 consuming 240 L/diesel/ha for six cycles	11.472
Transport for processing*	Tractor Agrale Deutz BX 4150 coupled with wagon and consumption of 12.8 L of diesel for 8 h per day	0.612
Fertilization application		
N	Applied in a rate of 60 kg/ha/year	3.69
P_2O_5	Applied in a rate of 70 kg/ha/year	0.675
K_2O	Applied in a rate of 70 /ha/year	0.47
Fabrication and maintenance of farm machinery 3		2.27
Total fossil energy input for field operations:		20.21
Energy in production of napier grass (18 tons of biomass per hectare) and the arithmetic mean of five cycles x 16 GJ per ton of biomass		288
Energy balance (energy gained/energy invested)		14.6

of cellulose, it was observed in the two studied genotypes values very close to 30%. The calorific value of the biomass of the studied genotypes also did not vary significantly. A calorific value of 3,876 kcal per kg of biomass was associated with the genotype Roxo and 3,796 kcal per kg was associated with the genotype Gramafante.

In studies on elephant grass grown for forage purpose, the fiber content of the whole plant increased and protein content decreased, the longer the plant remains in the field (Andrade et al., 2005). The levels of fiber in the present study were similar to those reported by Savioli et al. (2000) and Campos et al. (2002), which observed values close to 40%, and by Queiroz Filho et al. (2000), who reported values up to 43%, for plants grown for 100 days in the field. According to McKendry et al. (2002), the levels of lignin and fiber observed in the genotypes used in the present study are considered satisfactory for energy production by direct combustion. There was no statistical difference for cellulose contents in genotypes with values ranging from 29 (genotype Roxo) to 31% (genotype Gramafante). The results obtained so far, together with the work of Quesada (2005) and Morais et al (2009, 2013), indicate that this species has good adaptability to soils with low fertility and shows that the N accumulated is partially sustained by the significant contribution of biological nitrogen fixation (BNF) in elephant grass.

Energy balance

The energy efficiency calculations resulted in an energy balance of 15:1. This means that for each unit of energy used in the process of biomass production, 15 units of energy are produced (Table 3). These results corroborate with Samson et al. (2005), who found an energy balance of 21:1 in the production system. These results show the high potential of elephant grass use for bioenergy. Studies on other species have also been performed; these include work of Schemer et al. (2008). The authors' studies on *Panicum virgatum* were based on an average yield of 8.15 Mg. ha^{-1} and obtained an energy balance of 5.4:1. This yield is about 60% lower than that obtained in the present study in which average dry matter yield in three years was 18 Mg. ha^{-1}. The energy balance obtained in the present study was 14.6 and in studies carried out by Schemer et al (2008), it reached 5.4. Danalatos et al. (2007) working with *Miscanthus Sinensis* under two doses of N fertilizer (50 and 100 kg of N ha^{-1}), observed that there was no significant response to these treatments, obtaining an average yield of 27 Mg. ha^{-1} of DM in 270 days of cultivation. Quesada (2005) found values of up to 30 Mg ha^{-1} of DM in eight months of

cultivation with genotypes Cameroon and Gramafante without the application of N-fertilizer.

Conclusions

Gramafante cultivar is the most promising for energy production purposes. The energy balance of elephant grass biomass production under the experimental conditions was approximately 15:1, showing its high potential for use in bioenergy through direct burning.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Development of liquid inoculants for strains of *Rhizobium tropici* group using response surface methodology

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Common bean (*Phaseolus vulgaris* L.) is the most important legume for human consumption in many countries of South and Central America, Asia and Africa. The crop can benefit from the biological nitrogen fixation process, especially when inoculated with elite rhizobial strains. Strains belonging to the "*Rhizobium tropici* group" are preferred because they show high tolerance to abiotic stresses, but their survival in liquid formulations is poor, limiting their use by farmers. In this study, response surface methodology (RSM) was used to develop liquid formulations for the commercial strains CIAT 899 (*R. tropici*) and PRF 81 (*Rhizobium freirei*). A significant interaction between dibasic potassium phosphate (K_2HPO_4) and yeast extract was observed, and to reach higher cell concentration, one should employ low concentrations of yeast extract and high concentrations of K_2HPO_4. A basic formulation which may represent the basis for the development of liquid inoculants for the common bean crop was developed.

Key words: Biological nitrogen fixation, inoculation, common bean, *Phaseolus vulgaris*, *Rhizobium freirei*.

INTRODUCTION

Brazil is today the world's largest common bean (*Phaseolus vulgaris* L.) producer and consumer and the crop has great economic and social importance not only to the country, but also other South and Central America countries, African and Asian countries. In Brazil, the grain production increased by 36% in 2016/17, with 3,418.3 thousand tons produced mostly by small farmers (CONAB, 2017). Nitrogen (N) is the nutrient most required

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by the common bean crop, with about 50% being exported by the grains (Araujo et al., 1996; Fageria and Baligar, 2005). N-fertilizers are traditionally applied to the crop, resulting in high cost and environmental pollution; however, the legume can take advantage of the biological nitrogen fixation (BNF) process. Rhizobia selection programs performed in Brazil have identified elite strains that can fully supply the plant's N needs (Hungria et al., 2000, 2003). The strains used in commercial inoculants in the country belong to the "*Rhizobium tropici* group", which includes rhizobial species that share a very similar symbiotic plasmid (Ormeño-Orrillo et al., 2012; Gomes et al., 2015). These species are highly adapted to typical tropical conditions, such as tolerance to high temperature and acidic soils; therefore, the strains are employed in inoculants for the common bean crop not only in Brazil, but also in other countries of South America and Africa (Gomes et al., 2015).

Peat has been known as "the golden" pattern carrier for rhizobial inoculants, due to its properties of richness in organic matter and nutrients, capacity of retaining moisture and to offer some protection to the bacteria against high temperatures and desiccation, enabling bacterial survival for longer periods (Singleton et al., 2002; Hungria et al., 2005; Lupwayi et al., 2005; Fernandes Júnior et al., 2009; Deaker et al., 2016). Nevertheless, peat presents great variability in its physical and chemical properties, besides being a non-renewable natural resource. On the contrary, liquid formulations allow high cell concentration void of contaminants and are preferred by farmers due to ease in application of seeds or in-furrow. However, cell viability both in the inoculant and at the field may be reduced in liquid formulations due to the lower protection against abiotic stresses (Singleton et al., 2002; Hungria et al., 2005; Lupwayi et al., 2005; Tittabutr et al., 2007; Fernandes Júnior et al., 2009; Yates et al., 2016). Particularly, for the *R. tropici* group, cell survival in liquid formulations has been critical; therefore, inoculants for the common bean crop are practically entirely peat-based, but an increased adoption by the farmers would be achieved if liquid inoculants are available.

The yeast extract-mannitol (YM) culture medium, developed almost a century ago (Fred and Waksman, 1928), has been broadly used to grow rhizobia, and slight modifications have been proposed through the decades (Vincent, 1970; Somasegaran and Halliday, 1982; Hungria et al., 2016). Basically, the medium has few components, of which the two main ones are mannitol as carbon (C) source and yeast extract. At industrial level, as mannitol is an expensive C source, it is usually replaced by glycerol or sucrose (Balatti and Freire, 1996; Menéndez et al., 2014).

An increase in the adoption of common bean inoculation with elite strains can highly affect grain production and soil fertility, as well as contribute to the mitigation of greenhouse gases (Hungria et al., 2013); the availability of liquid inoculants for the crop is critical to achieve this goal. The objective of this study was to develop liquid formulations for strains of the *R. tropici* group, to be used as inoculants for common bean crops.

MATERIALS AND METHODS

Culture conditions

Stock cultures of *R. tropici* strain CIAT 899 (=SEMIA 4077, =CNPSo 142, =USDA 9030, =ATCC 49672, =BR 322) and *Rhizobium freirei* strain PRF 81 (=SEMIA 4080, =CNPSo 122, =IPR-Pv81) were grown in YM medium (Vincent, 1970) modified for mannitol content (Hungria et al., 2016) for the preparation of pre-inocula. The composition of the modified YM medium consisted of (g L^{-1}): mannitol (5 g) (Synth®), yeast extract (0.4 g) (Acumedia®), dibasic potassium phosphate- K_2HPO_4 (0.5 g) (Anidrol), magnesium sulfate heptahydrate- $MgSO_4.7H_2O$ (0.2 g) (Vetec), sodium chloride-NaCl (0.1 g) (Vetec), and pH adjusted to ~6.8. Cultivation was carried out with shaking at 180 rpm and 28°C for 24 h until a concentration of 10^6 CFU mL^{-1} was reached. The cultures were diluted with the medium culture for the proper concentrations of the assays, based on the OD_{600} of the culture, previously obtained and adjusted according to the cell counting number. The experiments were carried out from the pre-inocula with an initial concentration of 10^4 CFU mL^{-1}.

Evaluation of different sources of carbon

Sucrose (Anidrol), glycerol (Invitrogen®) and glucose (Biotec®) were evaluated as alternatives C sources to mannitol, as they are also used by strains of the *R. tropici* group (Dall'Agnol et al., 2013). Although, sucrose is used in a lower extent by some species of the *R. tropici* group, including *R. freirei* (Dall'Agnol et al., 2013), both sucrose and glycerol were chosen because of their low cost. Glucose was evaluated because it is one of the two monosaccharides constituents of sucrose, representing a C-source promptly available for *Rhizobium*, not requiring hydrolysis to be consumed by the microorganism. The culture media were used for the cultivation of both strains.

All C sources were used at the concentration of 5 g L^{-1}. The tests were performed in triplicate in a volume of 100 mL culture medium. The pH of the media was adjusted to ~6.8 and growth was performed on an orbital shaker at 180 rpm and 28°C for 96 h. In order to avoid the caramelization process and the Maillard reaction, a concentrated glucose solution of 50 g L^{-1} was filtered on a nitrocellulose membrane (Merck Millipore™) with a porosity of 0.22 µm and added under aseptic conditions, to the flasks where glucose represent the C source.

Viable cell concentration of *R. tropici* strain CIAT 899 and *R. freirei* strain PRF 81 was obtained by serial dilution in modified YM medium (Hungria et al., 2016), with the drop-plate method (Miles et al., 1938), adapted as described by O'Hara et al. (2016). The results are expressed in CFU mL^{-1}.

Response surface methodology (RSM)

The data obtained in the carbon source experiments informed the decision to use sucrose as the main carbon source in the subsequent RSM studies. From the initial data obtained, a basic

Table 1. *Rhizobium tropici* CIAT 899 and *Rhizobium freirei* PRF 81 cellular concentration when growth was evaluated in medium with different carbon sources. Evaluation was performed after 96 h of growth.

Carbon source	*R. tropici* CIAT 899 (CFU mL$^{-1)}$	*R. freirei* PRF 81 (cfu mL^{-1})
Sucrose	1.50 10^9 ± 2.01 10^{8a1}	7.78E+08 ± 9.07 10^{7a}
Mannitol	1.33 10^9 ± 7.93 10^{7a}	7.33E+08 ± 1.67 10^{8a}
Glucose	8.15 10^8 ± 1.39 10^{8b}	1.16E+09 ± 1.91 10^{8a}
Glycerol	7.17 10^8 ± 6.11 10^{7b}	7.78E+08 ± 1.05 10^{8a}
CV (%)	15.20	20.38

[1]Means of three replicates, and when followed by the same letter, for each strain, are not statistically different by the Tukey test ($p \leq 0.05$).

liquid formulation was defined for the response surface methodology (RSM) experiments. A factorial planning of 2^3 and three central points was established. The evaluated response variable was the cell concentration in CFU mL^{-1}. The linear model was evaluated with the data of 11 experimental points. Three factors (independent variables) were tested, with two levels each. In this way, C, N and P sources were analyzed simultaneously. The formulations tested varied in sucrose, yeast extract and K_2HPO_4. The pH was adjusted to ~6.8. Bacteria were grown at 180 rpm and 28°C for 72 h. The evaluation of rhizobial concentrations was performed in Petri dishes by the drop-plate method modified as described by O'Hara et al. (2016). Three experiments (A, B and C) were performed with different concentrations of the analyzed variables, as shown in Supplementary Tables S1 to S6.

Statistical analysis

The Statistica 7.0 program (Statsoft®) was used to analyze the results obtained by the RSM. The test of the model's lack of fit was evaluated through analysis of variance (ANOVA) at $p \leq 0.05$.

RESULTS

Rhizobium spp. growth with different carbon sources

Considering the YM medium with 5 g L^{-1} of mannitol (Hungria et al., 2016), when the C source was replaced by sucrose, cell concentration of *R. tropici* CIAT 899 was estimated at 1.5 × 10^9 CFU mL^{-1} after 96 h, similar to the concentration of 1.33 × 10^9 CFU mL^{-1} reached with mannitol (Table 1). However, regarding glycerol and glucose as C sources, significantly lower growth was observed (p≤0.05). *R. freirei* strain PRF 81 was used and grew with the four carbon sources tested, not showing statistic difference after 96 h of growth (Table 1). Therefore, the authors decided to continue studies on the viability of using the surface response methodology to design inoculant formulation only with strain CIAT 899.

Development of liquid formulations

Experiment A

According to the conditions established for the development of inoculant's liquid formulations for *R. tropici* CIAT 899 (Supplementary Tables S1 and S2), the cell concentration obtained ranged from 1.56 × 10^9 to 3.56 × 10^9 CFU mL^{-1} (Table 2). The response of variable presented small variation in the central points, inferring a good repeatability of the process. The response of surface analysis started by assuming that the investigated region was a linear function of the factors and estimated by the equation:

$$y = \beta_0 + \beta x_1 + \beta x_2 + \beta x_3 + \beta x_1 x_2 + \beta x_1 x_3 + \beta x_2 x_3$$

It was possible to gauge the coefficients of the model and none of the parameters (sucrose, yeast extract, K_2HPO_4 or their interactions) were significant (Table 3). The first-order model, adjusted for the coded variables could not be described. Analysis of variance (ANOVA) suggested that none of the factors had major effects; there was no interaction or evidence of the lack of fitness for the curvature in the response of the explored region. The explained percentage of variation (R^2) was 67%. Therefore, in Experiment A, no effects of the variables were observed, when the concentrations of sucrose, yeast extract and K_2HPO_4 present in the basic medium were analyzed. Based on these results, the studies were continued with a formulation in which the C source was represented by sucrose, denominated as YSac medium.

Experiment B

In the second factorial plan with the addition of central points (Supplementary Tables S3 and S4), the cellular concentration of *R. tropici* CIAT 899 ranged from 1.00 × 10^9 to 3.11 × 10^9 CFU mL^{-1} (Table 4). The repeatability of the process was considered good due to the small variation presented by the central points. The regression coefficients of the model were determined and none of the parameters evaluated (sucrose, yeast extract, K_2HPO_4 or their interactions) was significant (Table 5). The first-order model, adjusted for the coded variables, could not be described. No major effects were observed

Table 2. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment A) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (cfu mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[a]	-1	-1	-1	5	0.4	0.5	1.56 10^9
2[a]	1	-1	-1	25	0.4	0.5	1.78 10^9
3[a]	-1	1	-1	5	2	0.5	3.56 10^9
4[a]	1	1	-1	25	2	0.5	2.67 10^9
5[a]	-1	-1	1	5	0.4	2.5	1.81 10^9
6[a]	1	-1	1	25	0.4	2.5	3.11 10^9
7[a]	-1	1	1	5	2	2.5	2.78 10^9
8[a]	1	1	1	25	2	2.5	2.22 10^9
9[a]	0	0	0	15	1.2	1.5	3.11 10^9
10[a]	0	0	0	15	1.2	1.5	2.11 10^9
11[a]	0	0	0	15	1.2	1.5	2.00 10^9

Table 3. Regression coefficient and *p*-values obtained for the factorial planning with central points (Experiment A).

Factor	Regression coefficient	*p*-values
Mean	2.43 10^9	0.0001
Sucrose	8.75 10^6	0.9590
Yeast extract	3.71 10^8	0.0812
K_2HPO_4	4.38 10^7	0.7982
Sucrose x Yeast extract	-3.71 10^8	0.0812
Sucrose x K_2HPO_4	1.76 10^8	0.3328
Yeast extract x K_2HPO_4	-3.51 10^8	0.0933

with any of the factors, interaction between them, or no evidence of lack of fitness for the curvature, according to the analysis of variance. The explained percentage of variation (R^2) was close to 67% (Table 5).

In Experiment B, the concentrations of the C, N and P sources present in the YSac medium were defined as low (-1), five times higher in the central point (0) and 10 times higher in the high level (1) (Supplementary Tables S3 and S4). The proportions of C : N : P present in the YSac medium were maintained as the basis for optimization, but again, no main effects or interactions was observed.

Experiment C

Cellular concentrations of *R. tropici* CIAT 899 ranged from 1.56 × 10^9 to 4.56 × 10^9 CFU mL^{-1} after 72 h of growth (Table 6). The small variation in the central points of the variable's response also indicated a good repeatability of the process (Table 6). The regression coefficients of the model were measured and both K_2HPO_4 and the interaction of K_2HPO_4 and yeast extract were significant. The first-order model, adjusted for the coded variables, was described as follows:

$$y = 2.74*10^9 + 5.96*10^8 x_3 - 5.14*10^8 x_2 x_3$$

Results of the analysis of variance indicated that the factor K_2HPO_4 (p = 0.0239) represented the main effect, and statistically significant interaction between K_2HPO_4 and yeast extract (p = 0.0379) was verified (Table 7). There was no evidence of lack of fitness for the curvature in the response of the explored region, indicating that the response surface was satisfactorily described by the model. The explained percentage of variation (R^2) was close to 77%.

The response surface and the level curves obtained are shown in Figures 1 and 2. The plot of experimental values against predicted values showed that points were randomly distributed near the line, indicating good agreement and that the model did not show lack of significant adjustment (Figure 3).

Table 4. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment B) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (CFU mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[b]	-1	-1	-1	5	0.4	0.5	1.78 10^9
2[b]	1	-1	-1	50	0.4	0.5	1.00 10^9
3[b]	-1	1	-1	5	4	0.5	3.11 10^9
4[b]	1	1	-1	50	4	0.5	2.11 10^9
5[b]	-1	-1	1	5	0.4	5	1.89 10^9
6[b]	1	-1	1	50	0.4	5	1.89 10^9
7[b]	-1	1	1	5	4	5	1.89 10^9
8[b]	1	1	1	50	4	5	2.00 10^9
9[b]	0	0	0	25	2	2.5	2.67 10^9
10[b]	0	0	0	25	2	2.5	2.22 10^9
11[b]	0	0	0	25	2	2.5	1.89 10^9

Table 5. Regression coefficient and *p*-values obtained for factorial planning with central points (Experiment B).

Factor	Regression coefficient	*p*-values
Mean	2.04 10^9	0.0000
Sucrose	-2.09 10^8	0.1763
Yeast extract	3.19 10^8	0.0664
K_2HPO_4	-4.12 10^7	0.7621
Sucrose x yeast extract	-1.38 10^7	0.9192
Sucrose x K_2HPO_4	2.36 10^8	0.1370
Yeast extract x K_2HPO_4	-2.91 10^8	0.0840

DISCUSSION

Studies carried out by Ormeño and Zúñiga (1998) with the purpose of evaluating economic alternatives for YM medium, aiming at enabling the commercial production of inoculants, reported no significant difference in the growth of *Rhizobium* sp. PLC213, isolated from *Phaseolus lunatus*, when sucrose and mannitol were compared, while glycerol resulted in significantly lower growth. Similar results were obtained in the current study for *R. tropici* CIAT 899. However, *R. freirei* PRF 81 did not present significant differences in the comparison of the evaluated C sources.

It is worth mentioning that Castellane et al. (2014) observed higher growth and production of exopolysaccharides (EPS) when strain PRF 81 was grown in culture medium containing sucrose as the C source. Therefore, besides being a C source for *Rhizobium*, sucrose can favor the production of EPS, contributing to the protection against desiccation, by forming a layer with high water content around the cell, and favoring biofilm formation (Donot et al., 2012), altogether, leading to an increase in microorganisms survival in liquid formulations (Singleton et al., 2002; Taurian et al., 2010; Herrmann and Lesueur, 2013), and improving the inoculum quality. Other cellular components related to the C metabolism, such as polyhydroxybutyrate (PHB), can also help bacterial cell survival, improving inoculant quality (Tal and Okon, 1985; Santos et al., 2017).

The response surface methodology (RSM) consists of a combination of mathematical and statistical tools to delineate and analyze experiments for mathematical modeling of the responses (Box et al., 1978). The methodology allows the responses optimization, contributing to the improvement of products and processes, saving time and costs (Bas and Boyaci, 2007; Hao et al., 2011; Sütoa et al., 2015). When the RSM was applied in Experiment C, the response surface was shown as an inclined plane with respect to the right-to-left ascending axes and indicated that higher values of CFU mL^{-1} were obtained by moving the experimental region to

Table 6. Cellular concentration (CFU mL^{-1}) of *Rhizobium tropici* CIAT 899 obtained with the use of factorial design 2^3 (Experiment C) and three central points. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4). Growth was evaluated after 72 h.

Run	Coded variable			Real variable (g L^{-1})			Responses (CFU mL^{-1})
	x_1	x_2	x_3	C	N	P	
1[c]	-1	-1	-1	25	2	2	1.56 10^9
2[c]	1	-1	-1	45	2	2	1.56 10^9
3[c]	-1	1	-1	25	4.4	2	2.89 10^9
4[c]	1	1	-1	45	4.4	2	2.00 10^9
5[c]	-1	-1	1	25	2	5	3.00 10^9
6[c]	1	-1	1	45	2	5	4.56 10^9
7[c]	-1	1	1	25	4.4	5	3.00 10^9
8[c]	1	1	1	45	4.4	5	2.22 10^9
9[c]	0	0	0	35	3.2	3.5	3.00 10^9
10[c]	0	0	0	35	3.2	3.5	3.00 10^9
11[c]	0	0	0	35	3.2	3.5	3.33 10^9

Table 7. Regression coefficient and *p*-values obtained for factorial planning with central points (Experiment C).

Factor	Regression coefficient	*p*-values
Mean	2.74 10^9	0.0000
Sucrose	-1.38 10^7	0.9388
Yeast extract	-7.12 10^7	0.6937
K_2HPO_4	5.96 10^8	0.0239
Sucrose x yeast extract	-4.04 10^8	0.0744
Sucrose x K_2HPO_4	2.09 10^8	0.2825
Yeast extract x K_2HPO_4	-5.14 10^8	0.0379

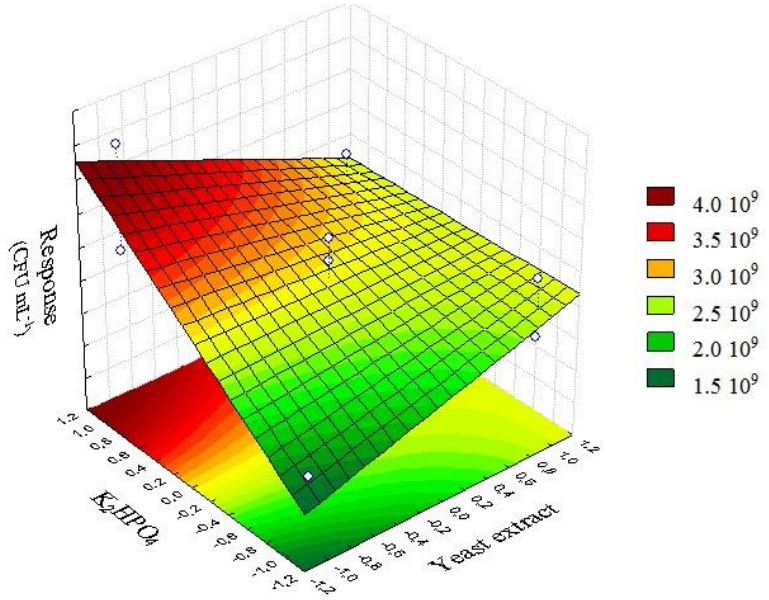

Figure 1. Response surface obtained for the factorial planning of 2^3 with three central points in Experiment C.

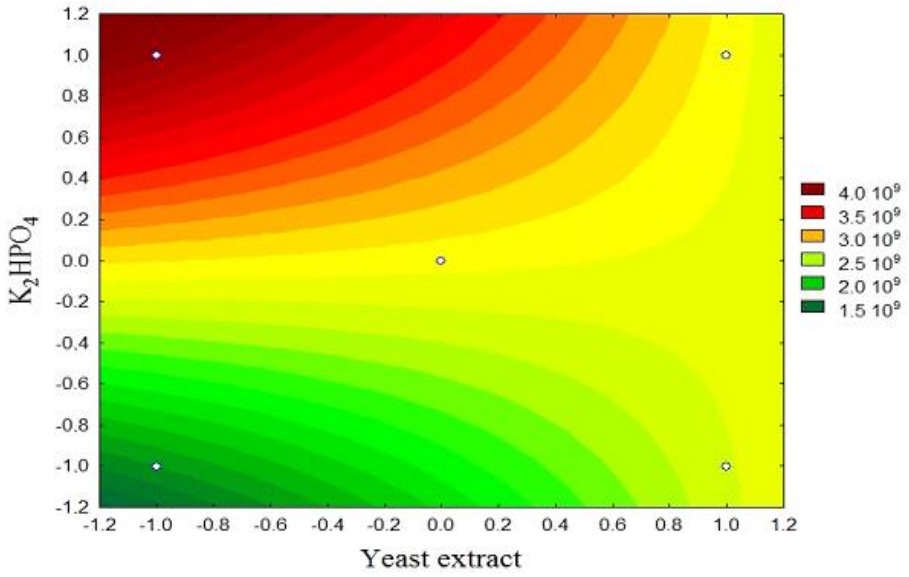

Figure 2. Level curves of the third factorial planning of 2^3 with three central points obtained in Experiment C.

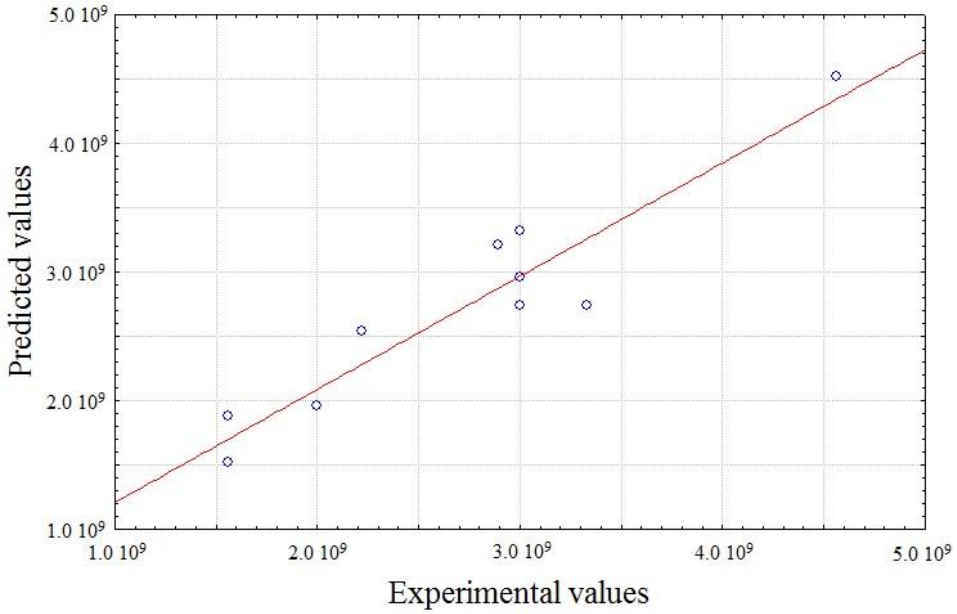

Figure 3. Experimental values plotted against predicted values for the model in factorial design 2^3 with three central points obtained in Experiment C.

lower values of yeast extract and higher values of K_2HPO_4 (antagonistic effect of factors). The results obtained in the present study have impact on the industry because the use of low concentration of yeast extract to obtain high cell growth rate is economically important, considering the high cost of this compound. In addition, there might be other benefits for the bacterium, as it has been observed for some species that the presence of high concentration of yeast extract (above 0.35%) may result in deformed cells and low viability (Skinner et al., 1977; Ben Rebah et al., 2007).

In Experiment C, higher concentrations of the three

factors were evaluated at lower level, in order to increase the cell concentration and to look for significant effects. Considering the RSM, the model $y = 2.74*10^9 + 5.96*10^8x_3 - 5.14*10^8x_2x_3$ would be used for the displacement. As factor 3 (variable K_2HPO_4) was the only one showing a significant effect according to the analysis of variance, the changes in the sucrose and yeast extract concentrations had no effect. The optimum values in the central point were determined. Recently, RSM was shown to be applicable in developing an inoculum from the plant-growth promoting bacterium, *Azospirillum brasilense* (Oliveira et al., 2017). From the current results, it can be concluded that the RSM can also be applied for the development of a basic liquid formulation for strains of the *R. tropici* group. The optimum values in the central point indicated the following concentrations: sucrose, 35 g L^{-1}; yeast extract, 3.2 g L^{-1} and K_2HPO_4, 3.5 g L^{-1}. This formulation may now be tested in the industry, validated at the field, have high impact on agriculture sustainability and increase the adoption of inoculation with elite strains by farmers growing common bean.

Conclusion

The response surface methodology (RSM) was used for the development of a liquid formulation for strains of *R. tropici* group, and to show that bacterial growth optimization required low concentrations of yeast extract and high concentrations of K_2HPO_4. A basic formulation that can impact the adoption of inoculation of the common bean crop was obtained.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Response surface methodology: Experiment A, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	5	15	25
Yeast extract (g L^{-1})	X_2	0.4	1.2	2
K_2HPO_4 (g L^{-1})	X_3	0.5	1.5	2.5

Table S2. Factorial planning of 2^3 (experiment A) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment A. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1a	-1	-1	-1	5	0.4	0.5
2a	1	-1	-1	25	0.4	0.5
3a	-1	1	-1	5	2	0.5
4a	1	1	-1	25	2	0.5
5a	-1	-1	1	5	0.4	2.5
6a	1	-1	1	25	0.4	2.5
7a	-1	1	1	5	2	2.5
8a	1	1	1	25	2	2.5
9a	0	0	0	15	1.2	1.5
10a	0	0	0	15	1.2	1.5
11a	0	0	0	15	1.2	1.5

Table S3. Response surface methodology: Experiment B, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	5	25	50
Yeast extract (g L^{-1})	X_2	0.4	2	4
K_2HPO_4 (g L^{-1})	X_3	0.5	2.5	5

Table S4. Factorial planning of 2^3 (experiment B) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment B. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1b	-1	-1	-1	5	0.4	0.5
2b	1	-1	-1	50	0.4	0.5
3b	-1	1	-1	5	4	0.5
4b	1	1	-1	50	4	0.5
5b	-1	-1	1	5	0.4	5
6b	1	-1	1	50	0.4	5

Table S4. Contd

7b	-1	1	1	5	4	5
8b	1	1	1	50	4	5
9b	0	0	0	25	2	2.5
10b	0	0	0	25	2	2.5
11b	0	0	0	25	2	2.5

Table S5. Response surface methodology: Experiment C, levels of sucrose, yeast extract and K_2HPO_4.

Variables	Parameter	Level		
		-1	0	1
Sucrose (g L^{-1})	X_1	25	35	45
Yeast extract (g L^{-1})	X_2	2	3.2	4.4
K_2HPO_4 (g L^{-1})	X_3	2	3.5	5

Table S6. Factorial planning of 2^3 (experiment C) with three central points for the development of liquid inoculant formulations containing *Rhizobium* spp. for the bean crop. Experiment C. C- C source (sucrose), N- N source (yeast extract) and P- P source (K_2HPO_4).

Run	Coded variables			Real variable (g L^{-1})		
	x_1	x_2	x_3	C	N	P
1c	-1	-1	-1	25	2	2
2c	1	-1	-1	45	2	2
3c	-1	1	-1	25	4.4	2
4c	1	1	-1	45	4.4	2
5c	-1	-1	1	25	2	5
6c	1	-1	1	45	2	5
7c	-1	1	1	25	4.4	5
8c	1	1	1	45	4.4	5
9c	0	0	0	35	3.2	3.5
10c	0	0	0	35	3.2	3.5
11c	0	0	0	35	3.2	3.5

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Full Length Research Paper

Determination of toxicity and chromatographic analysis of spilanthol content in *in vitro* culture of *Spilanthes oleracea* Jacq.

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***Spilanthes oleracea* Jacq. has been extensively used due to its pharmacological activities, and it has been considered as a promising vegetable crop. Nodal-segment cultures of *S. oleracea* were established in order to analyze the content of alkamide spilanthol. On average, 11 buds/nodal segment were developed after 30 days on MS basal salts liquid medium containing 2.22 µM benzyladenine (BA). Regenerated shoots formed complete plantlets in medium without growth regulators, and 80.2% of the regenerated plantlets survived in field conditions. Higher production of spilanthol (58.5% relative area) was obtained from the leaves and stems of plants grown in liquid medium up to 90 days, than in field-grown plants. Addition of BA to the culture medium significantly affected spilanthol accumulation. The production of spilanthol was also detected on the shoot basal callus. The spilanthol content varied with the plant organ in micropropagated plants. Ethanolic extract of leaves from field-grown plants showed no acute toxicology in Swiss mice at a dosage of 3 g/kg.**

Key words: *Spilanthes oleracea*, toxicity, plant regeneration, calli, spilanthol, gas chromatography.

INTRODUCTION

Spilanthes oleracea Jacq. is a plant of the Amazon region that is commonly utilized in local cuisine and folk medicine. The entire plant is claimed to have medicinal properties (Dubey et al., 2013). The leaves are eaten raw or as a vegetable by many tribes in India (Chakraborty et al., 2004) and also in the Amazon region. It is commonly

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known as “toothache plant” or “Paracress” (Wyk and Wink, 2009). The alkamide spilanthol, which occurs in several members of Asteraceae including *S. oleracea*, causes a pronounced tingling and mouthwatering effect upon ingestion. Industrial applications of this substance include oral care (Hirayama and Ikenishi, 2010), and as a flavoring and preservative in food (Miyazawa and Yamaguchi, 2010; Tanaka and Yagi, 2009). In cosmetics, it has been recently been employed as an anti-ageing ingredient, among other applications (Demarne and Passaro, 2008). Besides these applications, the leaf extract showed larvicidal activity against *Aedes aegypti*, increasing the possibility that it could be used as an important tool in the control of dengue (Ramsewak et al., 1999; Pandey et al., 2007, 2011).

The content of spilanthol is generally higher in the field-harvested flower heads, as also reported for *Echinacea* and *Spilanthes* (Perry et al., 1997; Nayak and Chand, 2002), than in other tissues, showing a tissue-specific distribution. Plant tissue culture can be used to induce quantitative and qualitative modifications in the production of plant secondary metabolites, by changing nutrient and hormone contents in the culture medium (Abyari et al., 2016; Collin, 2001). In addition, tissue culture eliminates the effect of climate conditions and diseases to which field-grown plants are subject.

Earlier studies of *in vitro* culture of different species showed that the accumulation of different secondary metabolites can be efficiently induced by various elicitors. Abiyari et al. (2016) demonstrated that the application of casein hydrolysate and L-phenylalanine is effective for productions of scopoletin. Alkamides were induced by methyl jasmonate (MeJa) in *Echinacea pallida* (Binns, 2001). Romero et al. (2009) demonstrated the efficacy of *E. pallida, Echinacea purpurea* and *Echinacea angustifolia* hairy root cultures in the *in vitro* production of alkamides. Moreover, *in vivo* elicitation through foliar application of elicitors [acetylsalicylic acid, salicylic acid, and methyl salicylate; as well as the metal elicitor (titanium (IV) ascorbate)] on *E. purpurea* increased the phenolic content up to 10 times as compared to the control, and also increased the biomass yield (Kuzel et al., 2009). Co-culture of different organs/species together also has been attempted since the co-culture provides the opportunity for metabolites produced by one organ/species to be excreted into medium and taken up by another organ. Sidwa-Goricka et al. (2003) have established co-culture of hairy roots of *Ammi majas* and cell-shoot suspension culture of *Ruta graveolens* to investigate possible interaction of metabolic pathways of coumarins, whereas, Wu et al. (2008) established the co-culture of ginseng (*Panax ginseng*) and *E. purpurea* adventitious roots for the productions of secondary metabolites.

This study aimed to assess spilanthol production in *S. oleracea in vitro* culture under the influence of benzyladenine (BA) and methyl jasmonate (MeJa), and evaluate the co-cultivation effect and acute toxicity of the crude extract from field-grown plants.

MATERIALS AND METHODS

Plant material and tissue culture

Seeds of *S. oleracea* Jacq. were collected in Belém, State of Pará, Brazil, and were identified by Dr. Ricardo Secco of the Emílio Goeldi Museum, Belém. Voucher specimens were deposited in the Emílio Goeldi Museum under catalogue number Herbarium MG 156.773. Four-week-old *in vitro* germinated seedlings were used as a source of explants for initiation of cultures in MS medium (Murashige and Skoog, 1962). Nodal segments excised from *in vitro*-cultured seedlings were inoculated in different treatments: culturing in the presence or absence of agar (30 and 90 days), under the influence of BA (2.22 and 4.44 µM).

The interaction with elicitors (2.0 µL salicylic acid and 45 µL methyl salicylate (Sigma Aldrich) added to ethanol, with a final concentration of 100 ppm (Binns, 2001) was put on cotton pieces that were put with the plants (with 60 days of culture on growth regulator-free MS medium). In addition, co-cultivation of nodal segments of *S. oleracea* and *Polygala paniculata* L. was performed. *P. paniculata* L. *in vitro* culture demonstrated production and release of methyl salicylate according to Victorio et al. (2011). The cultures were maintained in a growth chamber under cool-white fluorescent lighting tubes (1.6 W m^{-2}, 23 µmol.m^{-2} s^{-1} and daily photoperiod of 16 h at 25 ± 2°C).

Spilanthol extraction and GC analysis

Freeze-dried leaves, shoots and calli were macerated for 2 days in 90% chloroform (p/v) according to Simas (2003). The extract was then filtered and the solvent volume was reduced in a rotary evaporator. The resulting dry residue was weighed to determine the yield of crude extract for each treatment (Table 1).

GC-FID

Quantitative analysis of the extracts (30 mg/mL) was performed in a gas-chromatography system (Shimadzu GC-17A) equipped with a flame ionization detector (FID) and DB-5 capillary column (30 m, 0.32 mm, 0.25 µm), and 1 µL of each sample was injected with a split-mode injector (1: 6) into a flow of hydrogen gas held constant at 1 mL/min flow rate. The oven temperature was programmed for an initial temperature at 100°C, increasing at 10°C/min up to 200°C, held for 20 min, then a second ramp-up of temperature at 3°C/min to a final temperature of 250°C, and held for 5 min. The temperatures of the injector and detector were held at 250 and 200°C, respectively. The percentage content of spilanthol was calculated by integrating the areas of the corresponding signals.

Qualitative analysis was performed in a mass spectrometer (Hewlett-Packard, model HP-5971 A) coupled to a gas chromatograph, model HP-5890 A, Series II, equipped with a DB-5 capillary column (30 m, 0.32 mm, 0.25 µm). Experimental conditions were: ionization by electron impact at 70 eV, helium as carrier gas at flow rate of 1 mL/min. The National Institute of Standards and Technology (NIST, 1990) database was used for comparison of mass spectra.

Acute toxicology assay

Thirty female albino Swiss mice (25-30 g), two months old, were obtained from the central animal house of the Microbiology Institute/UFRJ. They were housed in standard polypropylene

Table 1. Yield of dried chloroform extracts (%, m/DM) obtained from dry mass *in vitro* plants of *S. oleracea* for different treatments.

Treatments	Leaf	Shoot	Total
MS/30 days	10.5	10.5	21.0
MS/90 days	4.9	2.0	6.9
2.2 µM BA/30 days	9.0	10.0	19.0
4.4 µM BA/30 days	20.0	7.6	27.6
Co-culture/30 days	14.2	6.2	18.4
Acclimatized plants/30 days	7.7	4.6	12.3

m/DM- Total extract mass/total dried plant mass.

Table 2. *Spilanthes oleracea in vitro* development. Effect of liquid and solid media, BA concentrations and co-culturing with *Polygala paniculata.*

Solid medium	MS 30 days	MS 90 days	Co-culture MS 30 days	2.22 µM BA 30 days	4.44 µM BA 30 days
No. of shoots	1.0±0.2	1.7±1.1		1.6±0.6*	1.5±0.7*
No. of nodal segment	3.1±0.7	12.7±4.6		4.4±1.3*	4.5±2.1*
No. of buds	6.2±1.4	26.8±9.1		8.9±2.6*	9.2±3.9*
Elongation (cm)	3.0±0.7	16.1±3.0		2.1±0.8*	2.1±1.0*
Rooting (%)	100	100		70*	13.3*
Líquid medium					
No. of shoots	1.0±0	1.5±0.8	1.0±0	2.0±0.7*	1.3±0.5*
No. of nodal segment	3.9±0.5	12.2±3.0	3.9±0.4	5.7±1.7*	3.9±1.9
No. of buds	5.8±1.0	23.5±6.1	7.9±0.7	11.3±3.3*	7.7±3.6*
Elongation (cm)	2.9±0.9	16.6±0	4.9±1.1	2.4±0.6	1.9±1.0*
Rooting (%)	100	100	100	73.3	56.7*

Mean ± standard error (n=30) * p≤0.05 in relation to control at 30 days. Kruskal-Wallis test.

cages, five per cage, and kept under controlled room temperature (28 ± 2°C; relative humidity 50-55%) in a 12-h light cycle. The mice were given a standard laboratory diet and water *ad libitum*. Food was withdrawn 12 h before and during the experimental period. The leaf ethanolic extract from field-grown plant was dissolved in 10% DMSO and administered to the animals orally by gavage. Two doses of the ethanolic extract (300 and 3000 mg/kg) were tested through intraperitoneal administration. On the first day of treatment, the animals were observed for 3 h, for any behavioral changes or deaths. After ten days of treatment, the animals were anesthetized lightly with ether and killed by cardiac puncture, and their body and organs (liver and spleen) were weighed. All experimental protocols were approved by the institutional Animal Ethics Committee (IBCCF/UFRJ 036).

Statistical analysis

All experiments used a fully randomized block design. Each experiment consisted of 5 nodal segments/vessel and 6 replicates per treatment (plant growth regulator, elicitors). All the experiments were repeated three times. The data were subjected to one-way ANOVA, and mean values were compared by Dunnett's multiple comparison test or by the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test as post-test, using the software GraphPad InStat, version 6.01. Rooting percent data were tested for the significance of the difference between two percentages, at the 5% significance level, using the software Statistica for Windows, version 5.0. For the quantitative analysis of phytochemical compounds, the data were collected from two independent experiments, and are presented as the mean values.

RESULTS AND DISCUSSION

In vitro culture of *S. oleracea*

Liquid or low-agar concentration media increase the growth of certain species cultured *in vitro* (Casanova et al., 2008; Abdoli et al., 2007), which is caused by greater availability of water and nutrients (Debergh, 1983); however, *S. oleracea* showed no difference in *in vitro* shoot development on liquid or solid media (Table 2). Liquid cultures are suitable for bud development and shoot multiplication, with BA added to the culture medium. Consequently, liquid medium was used throughout this study.

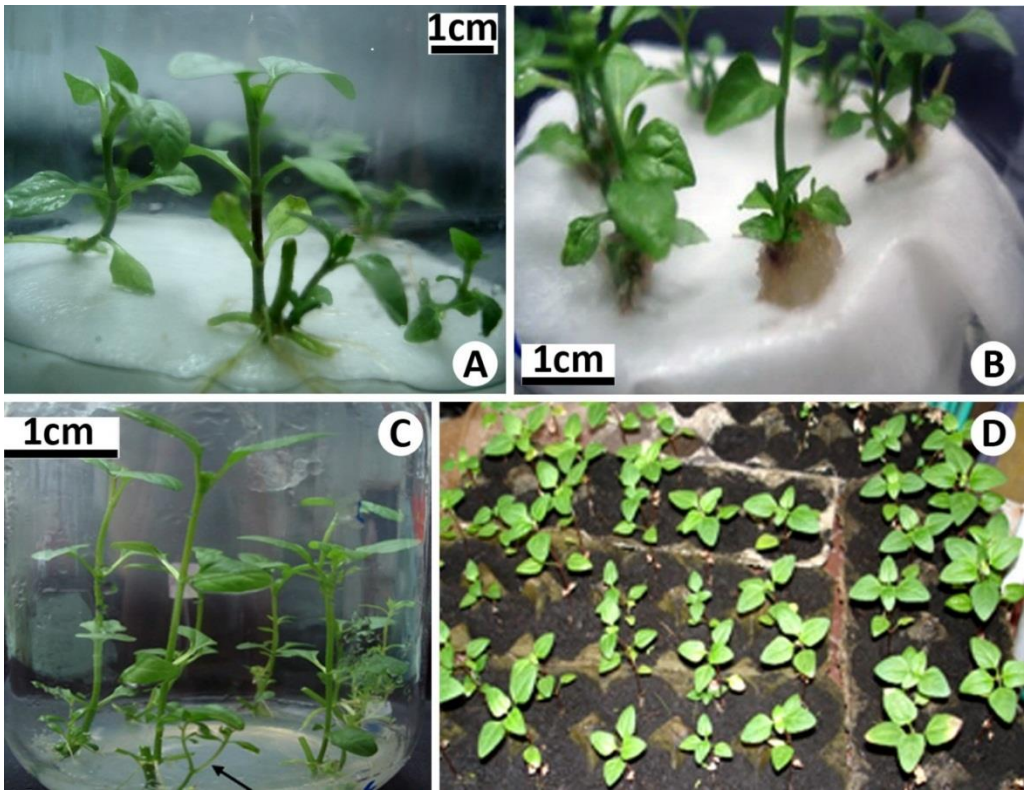

Figure 1. Plant regeneration from nodal segments of *S. oleracea***.** a- Shoot development from axillary buds induced by 2.22 µM BA liquid after 30 days. b- Basal and friable calli development in 4.44 µM BA liquid medium after 30 days. c- Co-culture of *Spilanthes oleracea* and *Polygala paniculata* (arrow); d- Acclimatized plants.

The growth ability of *S. oleracea* nodal explants was improved with the additional BA source (Figure 1a, b and Table 2) as previously reported (Haw and Keng, 2003; Saritha et al., 2002; Bais et al., 2002; Deka and Kalita, 2005; Saritha and Naidu, 2007). Furthermore, the assayed concentrations (2.22 and 4.44 µM) did not show significant differences in the explant responses except in relation to the root development. BA increased the multiplication rate (numbers of buds and nodal segments); however, rooting of BA-grown plantlets was inhibited, with no significant changes in shoot length. Basal-shoot calli were induced to the detriment of root development (Figure 1b). At the end of the cycle (multiplication phase), 70% of the plantlets had rooted. Auxin supplementation was not necessary for root formation.

The co-cultivation with *P. paniculata* (Figure 1c) resulted in an increase of the height of the plants, accompanied by increased bud neoformation. The significant difference in plant height between the co-culture and control reinforces the hypothesis that the methyl salicylate produced by *P. paniculata* may act as an allelopathic compound, since co-culturing stimulated the growth of *S. oleracea*. All plantlets acclimatized well, and the establishment of micropropagated plants occurred at a high rate (96%) (Figure 1d).

Gas chromatographic analysis of spilanthol content in micropropagated plants

Spilanthol content was determined in different parts of *in vitro* grown plants and in each tissue type analyzed (Table 3, Figure 2). The roots from *in vitro* plants, including acclimatized plants, contained no spilanthol (Table 3). Franca et al. (2016) determined the spilanthol content in all organs of *in vitro* culture of *Acmella oleracea* using Murashige and Skoog as basal medium and phytagel as the gelling agent. The aerial parts (leaves and stems) of plants exposed to 100 ppm salicylic acid and methyl salicylate did not contain spilanthol. However, the plants co-cultivated with *P. paniculata* maintained their capacity to synthesize spilanthol, with about 11.9% relative abundance (RA) (Table 3). Possibly, the concentration of elicitors may be related to the difference in responses between experiments involving methyl salicylate alone and methyl salicylate released from *P. paniculata.*

In order to increase the levels of spilanthol *in vitro*, Binns (2001), using elicitors such as jasmonates, found

Table 3. Values of relative area (%) of spilanthol content from plant organ extract for each treatment.

Treatments	Relative area (%)				
	Leaf	Stem	Root	Callus	Total
MS/30 days	8.3	10.7	0	-	19.0
MS/90 days	24.0	34.5	0	-	58.5
2.2 µM BA/30 days	14.7	20.5	0	2.1	37.3
4.44 µM BA/30 days	20.7	12.4	0	6.0	39.1
Co-culture/30 days	8.2	3.7	0	-	11.9
Acclimatized plants/30 days	2.4	1.7	0	-	3.9
100 ppm salicylic acid/24 h	0	0	0	-	0
100 ppm Methyl Salicylate/24h	0	0	0	-	0

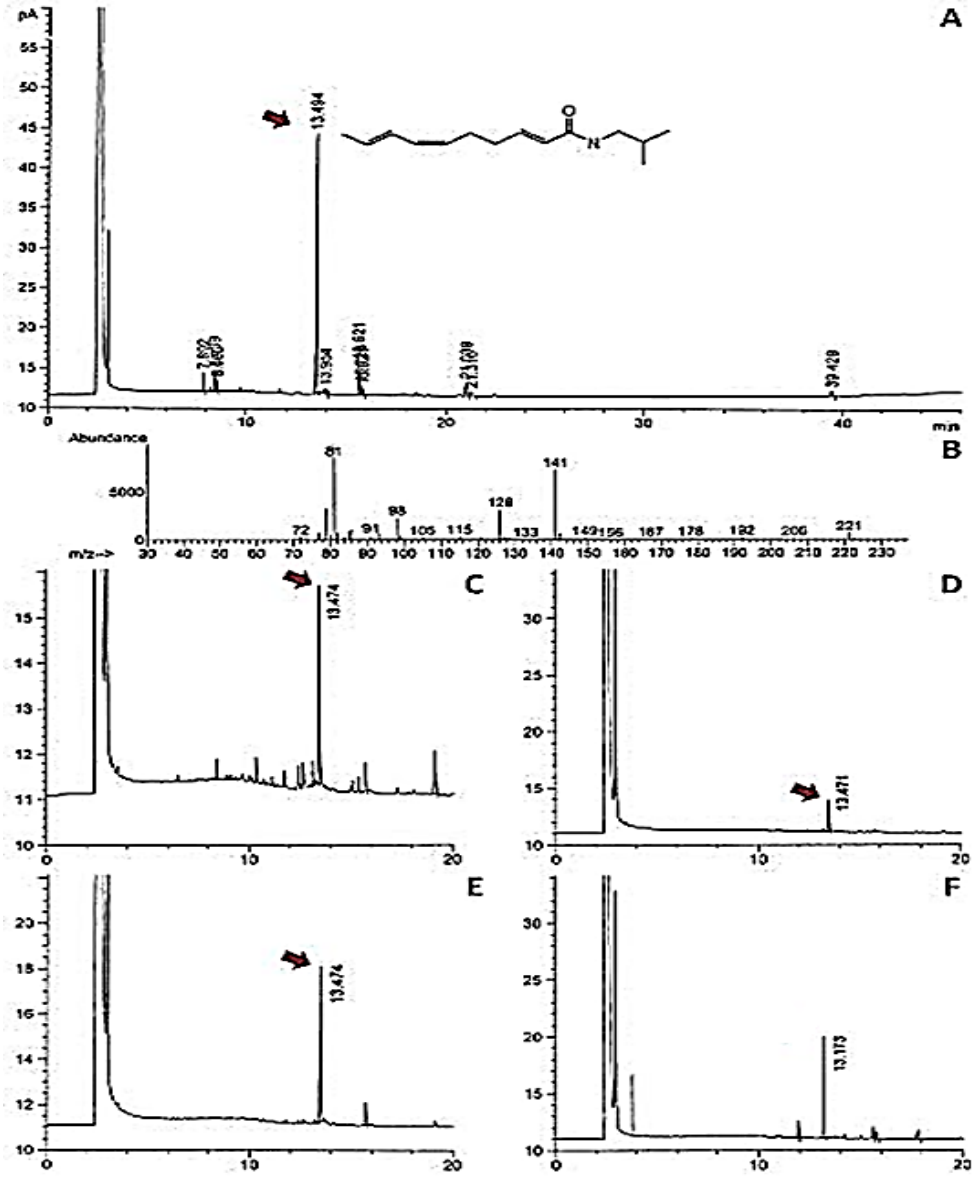

Figure 2. GC fingerprint of plant materials of S. oleracea. A- Chromatographic profile of field grown flowers of S. oleracea. B- Spilanthol fragmentation pattern. C to F- chloroformic extracts of samples from in vitro culture. C- leaves, D- Calli obtained from 4.4 µM BA, E- Shoot, F- Root.

Table 4. Mean weights (g) of mouse organs obtained from animals used (n = 5/treatment) for acute toxicology testing. The inoculum applied was an ethanol extract of aerial parts of field-grown plants of *S. oleracea*.

Inoculum	Spleen	Liver
	Median±standard deviation	
Control	0.145 ± 0.04	1.140 ± 0.10
DMSO (control)	0.164 ± 0.02	1.168 ± 0.30
Field plants 300 mg/kg	0.146 ± 0.05	1.202 ± 0.30
Field plants 3000 mg/kg	0.153 ± 0.04	1.295 ± 0.10

that methyl jasmonate increases the production of some alkamides in *Echinacea pallida*. However, in this study, the MeSa and SA treatments showed an inhibitory effect on spilanthol content at the concentrations assayed. The aerial parts of plants exposed to 2.22 and 4.44 μM BA had total spilanthol contents of 37.3 and 39.1% RA, respectively (Table 3). Basal calli grown in the medium exhibited spilanthol contents increasing from 2.1 to 6.0% RA in 4.44 μM BA (Figures 1c and 2). The highest spilanthol accumulation was found in MS control plants after 90 days of culture, reaching 58.5% RA of total content, against 19.0% in a 30-day culture (Table 3). However, when these highest-accumulating spilanthol plants were transferred to field conditions, the spilanthol content was low (4.0%) (Table 3).

Extracts from flowers enriched with spilanthol contained 1.2% in the pentane extract, 6.17% in the methanol extract, and up to 17% in the CO_2 supercritical fluid extract in different species of field-grown *Spilanthes* (Stashenko et al., 1996, Ramsewak et al., 1999). Data for spilanthol content specifically in leaves and stems are lacking, and the existing reports emphasize the contents in flowers or homogenized aerial parts of *S. oleracea*. However, Stashenko et al. (1996) calculated a spilanthol content of 21% as measured by area percent of gas chromatogram in a CO_2 supercritical fluid in an extract from leaves of *S. americana*.

The *in vitro* culture of *S. oleracea* showed the influence of nutrient-enriched Murashige and Skoog medium cultures on spilanthol accumulation. This accumulation increased in the time-consuming medium at 90 days of culture, and organ-specific production of spilanthol in the leaves, stems and even in the callus was observed. The results show the importance of *in vitro* MS culture, as compared to field culture, which did not result in spilanthol accumulation. In *in vitro* culture, *S. oleracea* had high spilanthol content in three months, from small amounts of *in vitro* plants extracted by maceration in chloroform solvent.

Acute toxicology assay

After ten days of the experiment, all animals treated intraperitoneally with the ethanol extracts of *S. oleracea* leaves (300 and 3000 mg/kg) were alive, with no external physical abnormalities. No notable changes were observed in the weights of liver and spleen from animals treated with the extract, in any dose tested, indicating no acute toxicity (Table 4). Chakraborty et al. (2004) conducted experiments with intraperitoneal administration of an aqueous extract of aerial parts (100, 200 and 400 mg/kg) for four hours, to evaluate the analgesic activity in albino mice. At the same time, the authors evaluated the acute toxicity of an aqueous extract of *S. acmella,* and observed no adverse effect or mortality in albino mice that ingested up to 3 g/kg *p. o*. during a 24-h observation period.

Spilanthol is one of several compounds that are extractable by ethanol from *S. oleracea* leaves. This possibility was demonstrated by Molina-Torres et al. (1999) in their spilanthol extraction and purification procedure from an ethanol extract. Ethanol, as used in this study, proved to produce a *Spilanthes* extract, which proved to have a safe composition in the animal-model tests. The absence of subchronic toxicology was observed in other studies, as related by Zuluaga et al. (2008) who administered an ethanolic extract of *Spilanthes americana* in Swiss albino mice, and by Ekor et al. (2005) who evaluated an aqueous extract of *Spilanthes filicaulis*.

Conclusion

The *in vitro* propagated *S. oleracea* in liquid-medium culture maintained the capacity to synthesize spilanthol in the leaves, stems and calli. The roots of *in vitro*-grown plantlets contained no spilanthol. Spilanthol was present in the regenerated plants; however, in much lower amounts than in *in vitro* culture. In this study, the plant organ-specific biosynthesis of spilanthol and *in vitro* culture proved to be an efficient method to obtain spilanthol in a liquid medium, without the need for the time-consuming addition of a growth regulator. The ethanolic extract obtained from field-grown leaves was proven to be safe, by an acute test in mice. The regeneration protocol and GC analysis developed here provide a new approach towards quality control of micropropagated plants. This method of producing

secondary metabolites has significant implications for the production of standardized-quality phytopharmaceuticals through mass production and analysis of the active ingredients.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Preliminary studies on the laxative properties of *Senna alata* L. and Hollandia yoghurt[tm]

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Experiments on *Senna alata* aqueous extract and Hollandia yoghurt were done to determine active compounds responsible for their laxative properties and to further establish sample vulnerability to microbial attack. Phytochemical screening was performed on test substrates to analyze for alkaloids, flavonoids, tannins, saponins, terpenoids and steroids. *Escherichia coli* (Gram negative), *Micrococcus* species (Gram positive), *Klebsiella pneumoniae* (Gram negative), *Enterococococcus* species (Gram negative) and *Salmonella* species (Gram negative) were test bacteria while *Penicillium* species, *Trichophyton* species, *Rhizopus* species, *Fusarium oxysporium* and *Aspergillus niger* were test fungi used for microbial assays. The results show that alkaloids, tannins, saponins, terpenoids and steroids were found in *S. alata* while only alkaloids, terpenoid and steroid were found in Hollandia yoghurt. *S. alata* had activity against *Penicillium* spp., *Trichophyton* spp. and *Rhizopus* spp., while Hollandia yoghurt showed no antifungal activity. Aqueous extract of *S. alata* and Hollandia yoghurt are however together active against test bacteria of which, *K. pneumonia* is common.

Key words: Laxative, *Senna alata*, hollandia yoghurt.

INTRODUCTION

Higher plant products have attracted the attention of microbiologists to search for some phytochemicals that may be linked to their exploitation as antimicrobials as it is felt that such plant products would be biodegradable and safe to human health (Kumar et al., 2008). Similarly, some proprietary food products on market shelves could attract further enquiries on their potency and capacity to improve human health. *Senna alata* L. has already been recognized as a potent laxative (Ogunti and Elujobi, 1993; Gritsanapan and Mangmeesri, 2009; Sule et al., 2010) and by experience, Hollandia plain sweetened yoghurt drink (www.chihollandia.com - NISISO 9001-2008; NIS ISO 22000-2005; NAFDAC Reg. No. 01-7506) has been found to induce bowel movements. Aqueous extract of *S. alata* obtained wild from Bowen University campus, Iwo was therefore subjected to biochemical and

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microbial analysis when compared with Hollandia yoghurt so as to determine their commonalities and differences. This study essentially investigated *in vitro* activities of extracts of the leaf of *S. alata* and Hollandia yoghurt on some fungi and bacteria isolates.

Several plants and seeds have been used to treat constipation; these includes the mature stem of *Neoboutonia velutina* Prain which was found to be effective for loperamide-induced constipation in rats (Ateufack et al., 2017) as well as peanut sprout ethanolic extract (Seo et al., 2013). While Zhang et al. (2013) suggested that bowel movement frequency and laxative use have no association with colorectal cancer (CRC), a more recent study by Citronberg et al. (2014) proved that non-fibre laxative use in the treatment of constipation increases CRC while the use of fibre laxative decreases it. *S. alata* L. has been credited for the treatment of hemorrhoids, constipation, inguinal hernia, intestinal parasites, blennorrhagia, syphilis and diabetes (Abo et al., 1998; Adjanahoun et al., 1991). The leaves of this plant were reported to be useful as purgative but were especially useful in treating dermatophytosis (Ogunti and Elujobi, 1993). *Senna* laxative properties were also examined by Izzy et al. (2016) in geriatrics in comparison with sorbitol, lactulose among other laxatives and were discovered to have a higher efficacy together with a very good adverse effect profile. In a study by Owoyale et al. (2005) in Kwara State Nigeria, *S. alata* was established to have antifungal activities. *S. alata* (L.) Roxb. is a medicinal plant that its leave extract has long been used as a laxative and antifungal drug (Gritsanapan and Mangmeesri, 2009). The leaves were said to contain anthraquinones with varying content, cultivating locations and harvesting period. *Senna* is also used for irritable bowel syndrome, hemorrhoids and weight loss. It contains many chemicals called sennosides that irritate the bowel lining and thus causing laxative effect (Therapeutic Research Faculty, 2009). Makinde et al. (2007) already established antifungal potency of alcohol extract of *S. alata* L. against certain fungi including *Trichophyton mentagrophytes, Candida albicans, Aspergillus flavus, Microsporum canis, Blastomyces dermatitidis* and also against some bacteria with maximum activity in fractions containing alkaloid salts and base.

MATERIALS AND METHODS

Source and identification of *S. alata* plant

Crude leaves of *S. alata* plant utilized in this study were obtained from Bowen University, Osun State, Nigeria and identified at the plant taxonomy laboratory, Department of Biological Sciences of the same university.

Test extracts

Extract of *S. alata* was obtained by soaking freshly washed green leaves in distilled water for three days. The leaves, after 72 h culture showed no growth which is an evidence of no contamination of the aqueous extract. The *S. alata* extracts (35 mg/mm) were filtered, poured in clean test tubes and stored in a refrigerator at 4°C (set was added as authors' own experimental measure).

Hollandia yoghurt (manufactured by Chi Ltd., Lagos, Nigeria), on the other hand, was sourced and purchased tetra-packed, aseptically stored in the refrigerator at 4°C until used.

Test fungi and bacteria

Five fungi and five bacteria used for this study were obtained from the Microbiology Laboratory of Bowen University. The test fungi were *Rhizopus* species, *Penicillium* species, *Fusarium* species, *Aspergillus niger* and *Trichophyton* species and the test bacteria were *Escherichia coli, Micrococcus* species*, Enterococcus* species, *Klebsiella* species and *Salmonella* species. The choice of these microbes was informed by known potentials and capabilities of causing intestinal tract infections (Joshua et al., 1992; Eistenstein and Zaleznick, 2000).

The fungi were obtained by sub-culturing to get pure isolates, before they were inoculated into enriched liquid medium containing ammonium chloride, magnesium sulphate, ferric citrate, dipotassium hydrogen phosphate, starch and yeast in distilled water.

The bacteria were confirmed by streaking on Eosin Methylene Blue agar; nutrient agar, thiosulphate citrate bile sucrose (TCBS) agar and *Salmonella-Shigella* agar producing green metallic sheen; tiny, yellow colonies; mucoid, raised and shiny colonies, and black precipitate, respectively (Isenberg, 2004). Each of the bacteria were inoculated into 5 ml sterile peptone water and incubated at 37°C for 18 to 24 h.

Pure isolates were later transferred onto sterile agar slants used for antimicrobial assay of both the aqueous *Senna* extract and Hollandia yoghurt.

Phytochemical screening

The aqueous test substrates were screened for their phytochemical bases using the standard method of Trease and Evans (1989) and Harborne (1998). The phytochemical components explored were alkaloids, tannins, saponins, flavonoids, terpenoids and steroids. The screening process and results obtained are shown in Table 1.

Antibacterial and antifungal activities

Antimicrobial activities of the *S. alata* extract and Hollandia yoghurt were determined using filter paper disc method (Jonathan et al., 2008). Whatman number 1 filter paper was cut into discs of 5 mm diameter before sterilization at 121°C for 15 min in an autoclave. After cooling, the sterile discs were soaked in the test extract and yoghurt sample and allowed to dry for about 5 min.

Mueller-Hinton agar (MHA) and potato dextrose agar (PDA) were aseptically inoculated with the test bacteria and fungi, respectively; using sterile swab sticks. The sample soaked discs were placed on seeded agar plates and left for about 10 min before incubation at 37°C for 24 h (for bacteria) and 27°C for 72 h (for fungi). Sterile water was used as control. Zones of inhibition were observed and measured metrically after incubation and the results are presented in Table 2.

RESULTS AND DISCUSSION

The results of the phytochemical analyses indicated the

Table 1. Phytochemical analyses of *Senna alata* extract and Hollandia yoghurt.

Phytochemical	*Senna alata*	Hollandia yoghurt
Alkaloids	+	+
Tannins	+	-
Flavonoids	+	-
Saponins	+	-
Terpenoids	+	+
Steroids	+	+

+, present; -, absent.

Table 2. Antimicrobial value of test substrates.

S/N	Bacteria	*Sienna alata*	Hollandia yoghurt	Fungi	*Sienna alata*	Hollandia yoghurt
		Zones of inhibition (mm)			Zones of inhibition (mm)	
1	*Enterococcus* spp.	-	10.0	*Aspergillus niger*	-	-
2	*Escherichia coli*	-	-	*Fusarium* spp.	-	-
3	*Klebsiella pneumoniae*	12.0	10.0	*Penicillium* spp.	5.0	-
4	*Micrococcus* spp.	12.0	-	*Rhizopus* spp.	8.0	-
5	*Salmonella* spp.	-	-	*Trichophyton* spp.	5.0	-

presence of alkaloids in *Senna* extract and the yoghurt which was confirmed by brown precipitate with turbidity; a green-black to blue coloration which indicated the presence of tannins in the *Senna* extract but not the yoghurt; the formation of stable foam that was taken as evidence of saponins in the *Senna* extract but not the yoghurt; a yellow coloration was observed for the *Senna* extract which shows the presence of flavonoid but no reaction for the yoghurt and reaction upper layers turned red and the sulphuric acid layer showed yellow with green fluorescence, indicating the presence of steroids in both the *Senna* extract and the yoghurt. These are in agreement with the submission of Sule et al. (2010) which revealed alkaloids, carbohydrates, saponins, anthraquinones, steroids and tannins as phytochemicals of *S. alata.* Egunyomi et al. (2009) already implicated the anthraquinones as acting on the gastro-intestinal tract to increase peristalsis action. By extension, Egunyomi et al. (2009) felt that *S. alata* may be useful as mild laxative especially in cases where patients complain of constipation. A color change from pink to violet also indicated the presence of terpenoids in both the *Senna* extract and the yoghurt.

Phytochemical analyses of the *Senna* extract uncovered important secondary metabolites including tannins, saponins, alkaloids, terpenoids, and steroids, while only the last three are present in Hollandia yoghurt. The commonality of alkaloids, terpenoids, and steroids in both *S. alata* and Hollandia yoghurt suggested that they may be active laxative constituents which should be further researched. Marked antibacterial activities of *Senna* extract were recorded for *Klebsiella pneumoniae* and *Micrococcus* spp., both having an average of 12 mm inhibition. Hollandia yoghurt was also reactive to *Enterococcus* spp. (10 mm inhibition) and *K. pneumonia* (10 mm inhibition), affirming that the two trial samples were active only against *K. pneumoniae* among the bacteria examined.

Hollandia yoghurt showed no antifungal activity, while *S. alata* extract inhibited *Penicillium* spp. (5 mm); *Rhizopus* spp. (8 mm) and *Trichophyton* spp. (5 mm). This means only bacteria resistance could be established as common to the trial samples and not antifungal resistance. Perhaps, the peristalsis improvement in the alimentary carnal may be attributed to bacteria reticence. The main bacterium implicated in this study is *K. pneumoniae* with minimum effective inhibition zone of 10 mm. Sule et al. (2011) also reported the antifungal activity of *S. alata* crude stem bark extract against *Trichophyton* spp. and other dermatophytes.

Conclusion

The phytochemical analyses revealed the presence of important secondary metabolites including tannins, saponins, alkaloids, terpenoids and steroids while only the last three are present in Hollandia yoghurt. The presence of these bioactive compounds as well as antibacterial properties of *S. alata* and Hollandia yoghurt provided insight into their usage for relieving constipation. These potentials may have applications against microbial

infections and diseases for therapeutic purposes and should be explored in the production of antimicrobial drugs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity study of Ethiopian Faba bean (*Vicia faba* L.) varieties based on phenotypic traits and inter simple sequence repeat (ISSR) markers

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Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes of the world. This study was designed to reveal the genetic diversity existing among 32 Ethiopian faba bean varieties grown at three locations (Sinana, Agarfa and Selka) using 23 phenotypic traits and 11 inter simple sequence repeat (ISSR) primers. The combined analysis of variance across the three locations showed highly significant (*p<0.05*) variations among the varieties for many of the traits. The un-weighted pair group method with arithmetic mean of phenotypic traits revealed five major clusters. Eleven ISSR primers amplified 120 bands, of which 107 loci were polymorphic. Primer 860 recorded the highest gene diversity (0.38) and Shannon index (0.56), while primer 848 and 857 exhibited the least gene diversity (0.18). Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) of ISSR primers data grouped the cultivars into three major clusters based on Jaccard's similarity coefficient ranging from 0.41 to 0.77. The principal coordinated analysis also categorized the varieties into three different groups similar to that of cluster analysis. The genetic variation in these cultivars suggests their potential use in faba bean breeding programs via introgression with other germplasm resources for faba bean improvement.

Key words: *Vicia faba*, genetic diversity, phenotypic traits, inter simple sequence repeat (ISSR).

INTRODUCTION

Faba bean (*Vicia faba* L., 2n = 12) is one of the oldest domesticated food legumes with controversial origin. Vavilov (1936) proposed that faba bean originated and was domesticated in the Central Asia and then expanded westward along the mountains to Iran, Turkey, the Mediterranean and Spain, while Cubero (1974) suggested

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that Near East, Iraq and Iran are the primary centers of origin and Afghanistan and Ethiopia are the secondary centers of origin. However, recent evidence from archeological study strongly suggests that Neolithic people in Israel (the lower Galilee: Ahihud, Nahal Zippori and Yiftah'el) first domesticated faba beans at least 10,200 years ago, and they were eating it as a staple food before grain was cultivated in the other areas (Caracuta et al., 2015).

Faba bean is one of major grain legume crops grown in Ethiopia and ranks first in terms of area in production with 574,060.45 ha and 943,964.2 tons produced (CSA, 2014). It is used both as food and feed due to its high protein content, and to enhance soil fertility (Caracuta et al., 2015). It is also used as a break crop to interrupt disease cycles and weed control for cereal-based crop rotations (Erik et al., 2012). Despite its ecological and economic contributions and the value of faba bean in Ethiopia, the productivity is still far below its potential of 1.9 ton ha^{-1}, due to chocolate spot, aschocyta blight, rust, drought, soil acidity and water logging (Asfaw et al., 1994; El-Fouly, 1982). To address these challenges, designing a sound crop breeding programs that would improve productivity of the crop through development of superior cultivars with stable performance across agro-ecologies plays an important role.

For such reason, documenting genetic information of this important crop is crucial. Characterization of genetic diversity study plays a paramount role in revealing genetic variability among and within species, developing selection criteria, selecting heterotic parents for hybridization, choosing effective breeding procedures and in determining conservation strategies (Singh, 1990). Estimation of genetic variation among faba bean varieties in Ethiopia is, therefore, the main concern of breeders as the effectiveness of selection depends on the proportion of the heritable variation.

Different marker systems such as morphological, biochemical and molecular markers are typically used to assess the genetic diversity of crops species. Morphological markers reveal genetic diversity of crops based on phenotypic appearance though it is highly influenced by environmental factors and can be more subjective than other markers (Vos et al., 1995; Hedrick, 2005; Li et al., 2009). Similarly, biochemical markers are low in abundance and polymorphisms, and are sensitive to environment. So, the limitations of both morphological and biochemical markers are addressed by the DNA based molecular markers. Several authors have reported the genetic diversity of faba beans using different molecular markers, including: SDS PAGE (Hou et al., 2014), random amplified polymorphic DNA (RAPD) (Basheer-Salimia et al., 2013; Aziz and Oman, 2015), amplified fragment length polymorphism (AFLP) (Zeid et al., 2001; Ammar et al., 2015), SRAP (Ammar et al., 2015) and inter simple sequence repeat (ISSR) (Terzopoulos and Bebeli, 2008; Abdel-razzak et al., 2012; Mejri et al., 2012; Wang et al., 2012; Salazar-Laureles et al., 2015). These markers have been instrumental in revealing genetic diversity within and among populations of faba bean genotypes.

Combining molecular markers with phenotypic markers is an important approach for varietal characterization and fingerprinting to reveal the relationship and level variability among faba bean varieties cultivated in Ethiopia. Genetic diversity of released Ethiopian faba bean varieties has not been studied yet, so, this research aimed to investigate the diversity and relationships between Ethiopian faba bean cultivars using both phenotypic traits and ISSR markers.

MATERIALS AND METHODS

Descriptions of the study area

The field experiment was conducted at three locations in southeastern Ethiopia highland, Sinana (07°07'N, 40°10'E), Agarfa (07°15'44''N, 039°50'38''E) and Selka (07°04'28''N, 040°12'18''E) during 2014/2015 for the analysis of agro-morphological performance, while ISSR analysis was carried out at Plant Genetics Research Laboratory, Addis Ababa University, Addis Ababa, Ethiopia. All the three experimental sites are characterized by pellic vertisol with a slightly acidic soil with altitude range of 2400 to 2509 m a.s.l that represent the potential production area.

Plant materials

A total of 32 faba bean varieties released from different research centers in the country were used for variability assessment using morphological traits and ISSR markers (Table 1). The field experiment was laid out using Alpha lattice design with two replications at three locations (Sinana, Agarfa and Selka). Each plot consisted of two rows 4 m long with a row-to-row spacing of 40 cm and plant-to-plant spacing of 10 cm. The genotypes were assigned to plots randomly within each replication. Fertilizer was applied at the rate of 100 kg ha^{-1} at planting. For ISSR genotyping, leaf samples were collected and dried with silica gel for DNA extraction. Silicagel dried leaf samples were ground with Mixer Mill (Retsch GmbH, Germany).

Genomic DNA extraction

Genomic DNA was extracted with a minor modification using cetyl trimethyl ammonium bromide (CTAB) method employing triple extraction to yield optimal quantities of high-quality DNA from tissues (Borsch et al., 2003). DNA quantity and quality were tested using gel electrophoresis and Nano drop spectrophotometry (Nano-drop 2000/2000c). Genomic DNAs were then diluted to approximate amount of 70 ng/µl for polymerase chain reaction (PCR) to screen primers and optimize the PCR reaction condition.

Primer selection and PCR amplification

Initially, a total of 25 ISSR primers (Primer kit of UBC 900) were selected based on published research report on faba bean (Abdul-Razzak et al., 2012; Mejri et al., 2012; Wang et al., 2012; Salazar-Laureles et al., 2015) and other related crops: chick pea (Bhagyawant and Srivastava, 2008), lentil (Edossa et al., 2007;

Table 1. Combined mean performance analysis of 23 traits of 32 Ethiopian faba bean varieties tested at Sinanaa, Agarfa and Selka during main growing season of 2014.

Variety name	LL	LW	LA	LAI	PL	PW	IL	PHFP	PH	NBPPL	NPPL	NSPL	NSPPod
Mosisa	9.12	4.45	28.92	189.64	7.27	1.33	5.77	40.83	149.77	1.40	32.13	80.20	2.50
Tumsa	9.28	4.58	30.50	238.97	7.34	1.37	5.96	57.17	153.73	1.40	25.00	61.87	2.45
Hachalu	9.33	4.73	31.26	227.51	8.44	1.52	5.98	48.70	155.80	1.60	25.17	70.73	2.78
Dosha	9.48	4.65	31.28	281.89	7.87	1.40	5.38	40.80	142.30	2.23	33.20	87.83	2.63
Gachena	9.58	4.74	32.33	228.49	8.88	1.73	5.82	48.03	153.33	1.47	24.27	63.83	2.64
Walki	9.53	4.97	33.58	247.96	6.90	1.28	5.88	40.00	151.57	1.47	36.63	90.37	2.52
Obse	8.97	4.31	27.77	204.34	9.51	1.54	6.67	45.87	155.13	1.60	19.63	56.97	2.88
Moti	8.80	4.25	26.71	220.49	8.13	1.35	5.66	44.77	148.13	1.93	26.33	75.60	2.91
Gabelcho	9.48	4.35	29.25	249.65	7.77	1.54	6.14	47.17	150.93	1.87	26.80	68.47	2.57
Adet Hanna	9.80	4.74	32.95	212.38	7.19	1.25	6.41	44.10	150.47	1.13	36.03	90.90	2.53
NC-58	9.18	4.33	28.34	187.58	6.25	1.17	6.41	39.20	147.77	1.40	41.07	107.57	2.62
Wayu	8.94	4.07	25.95	181.65	5.72	1.10	6.39	46.40	142.87	1.43	37.53	94.13	2.50
Degaga	9.28	4.63	30.35	233.97	7.01	1.34	5.92	42.73	156.27	1.77	44.90	120.80	2.67
Dagm	8.69	3.87	23.98	203.88	5.98	1.04	5.65	45.23	138.77	1.97	40.63	109.73	2.71
Holetta-2	9.53	4.64	31.39	226.88	7.33	1.38	5.50	46.83	152.20	1.73	37.03	99.37	2.67
Shalo	9.60	4.75	32.24	254.45	7.38	1.47	5.83	51.97	154.20	1.60	31.73	76.57	2.42
Tesfa	9.39	4.81	31.80	213.00	6.93	1.28	6.11	41.57	148.73	1.30	34.57	93.23	2.73
Mesay	9.24	4.35	28.54	194.48	6.92	1.32	6.21	37.13	154.07	1.17	32.90	93.37	2.89
Bulga 70	9.08	4.44	28.49	217.94	6.33	1.14	5.99	38.83	147.33	1.83	38.67	108.93	2.83
Kassa	9.57	4.54	30.97	204.04	6.45	1.20	6.39	42.80	149.10	1.67	43.47	104.97	2.39
Gora	10.24	4.92	35.37	283.35	9.87	1.58	5.40	44.03	151.73	1.53	24.53	67.67	2.71
CS-20-DK	9.00	4.10	26.25	228.62	6.84	1.21	5.60	44.43	147.50	1.90	30.93	87.10	2.81
Kuse	9.00	4.22	27.06	198.39	6.61	1.28	5.59	40.30	145.10	1.53	35.37	95.80	2.71
Dida'a	10.03	4.71	33.17	242.11	8.86	1.58	5.53	44.30	155.57	1.53	26.17	69.33	2.64
Lalo	9.34	4.04	26.81	181.38	6.22	1.17	5.34	46.63	142.33	1.53	34.17	90.50	2.65
Bako local	8.63	4.11	25.48	233.18	6.52	1.13	6.35	42.97	141.30	1.83	37.20	99.00	2.64
Debrebirhan local	8.73	3.70	23.24	213.76	5.57	1.05	6.01	38.40	135.60	2.23	47.60	123.20	2.59
Sinana local	9.18	4.58	29.86	212.57	7.04	1.32	6.09	39.93	150.53	1.27	35.03	89.07	2.54
Agarfa local	9.07	4.89	31.52	226.31	7.08	1.45	5.59	37.13	149.43	1.33	35.73	92.33	2.56
EKCSR-02006	9.03	4.44	28.38	197.81	7.32	1.39	5.88	38.70	143.83	1.53	34.83	81.13	2.33
Adet local	9.26	4.68	30.67	251.01	7.63	1.40	6.23	48.20	151.83	1.80	34.97	89.87	2.58
Kulumsa local	10.09	5.17	36.74	254.46	7.81	1.47	6.11	46.10	151.17	1.67	38.03	91.43	2.44
Mean	9.30	4.49	29.72	223.19	7.28	1.34	5.93	43.79	149.01	1.61	33.82	88.50	2.63
CV	6.10	9.13	13.38	23.11	6.75	12.35	11.42	14.70	5.40	25.54	20.30	21.04	8.24
LSD (5%)	0.35	0.34	4.01	42.45	0.59	0.20	0.64	5.68	4.65	1.31	8.34	20.40	0.15

** = Highly significant($p<0.01$); * = significant ($p<0.05$); CV = coefficient of variation; LSD = least significant difference; LL = leaf length (cm); LW = leaf width (cm); LA = leaf area (cm^2); LAI = leaf area index; Pl = pod length (cm); PW = pod width (cm); IL = internodes length (cm); HFPN = height to first poding node (cm); PH = plant height (cm); NBPPL = number of branches per plant; NPPL= number of pods per plant; NSPL= number of seed per plant; NSPPod = number of seed per pod.

Table 1. Contd.

Variety name	BMPP	SYPP	HI	TSW	DF	DM	SFP	SPE	EGR	CP
Mosisa	2966.67	4573.92	50.91	518.85	54.00	141.67	87.67	2891.92	53.98	22.90
Tumsa	3716.67	4260.07	36.57	620.47	55.67	147.83	92.17	2903.59	39.75	22.51
Hachalu	3583.33	4311.36	39.68	575.79	55.33	147.83	92.50	2836.00	37.14	20.98
Dosha	2683.33	4093.57	48.90	556.88	55.50	148.67	93.17	2639.31	26.54	22.07
Gachena	3850.00	4867.15	40.99	562.13	53.67	146.33	92.67	3242.05	43.05	21.36
Walki	3816.67	5631.46	43.81	558.09	53.00	143.67	90.67	3464.31	42.01	22.24
Obse	3350.00	4396.33	42.02	617.15	53.17	144.33	91.17	3020.01	36.25	21.29
Moti	3416.67	5256.44	41.79	583.93	51.83	147.33	95.50	3150.76	32.82	21.44
Gabelcho	3583.33	5113.17	38.34	524.65	56.00	144.17	88.17	2580.27	36.98	22.05
Adet Hanna	3116.67	4253.14	43.52	421.82	54.17	139.83	85.67	2611.61	46.30	22.38
NC-58	3000.00	4859.89	52.54	401.00	53.50	138.83	85.33	3090.95	47.99	22.29
Wayu	3300.00	4141.30	40.40	444.10	57.33	145.33	88.00	2461.21	45.97	21.95
Degaga	3400.00	5081.81	49.37	454.36	54.17	137.50	83.33	3040.16	52.69	22.56
Dagm	3166.67	3551.45	36.24	362.51	56.33	144.17	87.83	2137.32	31.53	22.92
Holetta-2	3575.00	4629.20	43.00	488.32	55.17	146.00	90.83	2938.92	37.93	20.59
Shalo	3750.00	4913.17	42.26	506.99	54.50	141.17	86.67	3003.48	51.81	22.65
Tesfa	3183.33	4339.87	44.09	411.39	55.33	138.33	83.00	2559.77	46.61	22.92
Mesay	3283.33	4393.18	42.24	406.32	54.67	140.83	86.17	2677.26	49.93	22.51
Bulga 70	2900.00	4283.07	47.79	412.81	54.50	141.83	87.33	2701.40	36.85	22.34
Kassa	3350.00	4678.05	44.85	412.42	53.00	142.50	89.50	3085.26	44.26	22.48
Gora	3500.00	4112.37	38.62	690.06	54.83	146.67	91.83	2660.75	37.28	22.87
CS-20-DK	3391.67	4469.20	42.15	504.47	55.50	145.33	89.83	2787.27	36.36	21.95
Kuse	3366.67	4905.53	47.27	470.99	53.67	142.33	88.67	3179.12	56.01	21.80
Dida'a	3616.67	4855.91	43.86	596.38	53.83	146.83	93.00	3351.94	40.14	22.63
Lalo	3216.67	3847.39	37.99	380.43	55.00	146.50	91.50	2430.73	32.72	22.12
Bako local	2616.67	2955.59	36.06	333.56	56.50	140.33	83.83	1716.27	26.67	22.33
Debrebirhan local	2583.33	3791.04	49.16	358.80	54.50	140.83	86.33	2357.21	39.68	22.79
Sinana local	3050.00	4737.69	50.21	406.84	53.33	140.17	86.83	2993.91	53.26	22.51
Agarfa local	3783.33	4286.42	44.34	519.28	52.33	142.33	90.00	3546.15	53.69	22.14
EKCSR-02006	3766.67	5305.15	45.53	509.26	54.00	147.00	93.00	3585.34	42.24	21.99
Adet local	3733.33	4608.15	39.97	520.38	54.00	147.00	93.00	3139.85	34.52	21.68
Kulumsa local	4200.00	4320.91	44.09	545.65	53.67	147.00	93.33	3862.48	42.98	20.95
Mean	3369.27	4494.44	43.39	489.88	54.44	143.77	89.33	2895.21	41.75	22.13
CV	17.45	7.20	15.84	14.09	2.18	1.45	2.50	9.16	28.02	3.29
LSD (5 %)	360.95	123.94	8.19	80.19	1.33	2.32	2.40	302.35	8.5	0.88

** = Highly significant($p<0.01$); * = significant ($p<0.05$); CV = coefficient of variation; LSD = least significant difference; SYPP = seed yield per plot(gm/m^2); HI = harvest Index; TSW =thousand seed weight (gram); DF = days to flowering; DM = days to maturity; SFP =seed filling period; SPE = seed production efficiency; SFR = seed filling rate; SYPD = seed yield per day; BMPR = biomass production rate; EGR = economic growth rate; CP = crude protein content.

Table 2. Combined analysis of variance for 23 traits of thirty-two Ethiopian faba bean varieties tested at Sinana, Agarfa and Selka during cropping season of 2014.

Traits	Loc (df=2)	Rep(loc) (df=3)	Variety (df=31)	Variety xloc (df=62)	Pooled error (df=93)	Mean	CV (%)	R^2
Leaf length (cm)	22.37**	0.22	0.91**	0.26**	0.09	9.32	3.26	0.91
Leaf width W (cm)	9.52**	0.40**	0.70**	0.14**	0.08	4.49	6.40	0.87
Leaf area (cm^2)	1108.08**	51.06**	59.38**	12.341**	10.21	29.72	10.75	0.84
Leaf area index	235538.98**	1329.23	4385.03**	3065.35**	1326.59	223.19	16.32	0.87
Pod length (cm)	0.96*	0.46	7.28**	0.3ns	0.24	7.28	6.78	0.91
Pod width (cm)	0.24**	0.07	0.17**	0.03ns	0.03	1.34	12.65	0.75
Internod length (cm)	27.54**	1.32	0.73**	0.51**	0.3	5.93	9.22	0.80
Plant height to first pod (cm)	4396.84**	24.60	118.54**	35.81*	22.78	43.79	10.90	0.87
Plant height (cm)	1380.68**	23.14	163.21**	42.12**	15.56	149.07	2.64	0.88
Numbers of branch per plant	2.48ns	1.08	1.57ns	1.44ns	1.20	1.69	25.68	0.74
Numbers of pods per plant	3081.89**	131.75	265.52**	53.14ns	51.61.00	33.82	21.24	0.79
Numbers of seeds per plant	23249.83**	42.41	1547.71**	328.94ns	284.56	88.49	19.08	0.81
Numbers of seeds per pod	0.13**	0.01	0.12**	0.05**	0.02	2.63	5.10	0.81
Biomass per plot (g/plot)	4189960.94**	65377.60	858732.78**	233697.5**	90807.71	3361.72	8.96	0.85
Seed yield per plot (g/plot)	3441447.89**	23325.12	179331.08**	888862.18**	10620.27	1438.22	7.17	0.95
Harvest index (%)	1590.82**	171.10	117.53**	62.55ns	44.90	43.39	15.45	0.73
Thusound seed weight (g)	288830.59**	547.00	46671.63**	17542.47**	4873.54	489.88	14.25	0.85
Days to flowering	649.83**	3.24	9.10**	5.05**	1.40	54.44	2.17	0.94
Days to flowering	482.92**	9.53	60.92**	33.95**	4.21	143.77	1.40	0.93
Seed filling period	188.26**	10.11	65.44**	32.92**	4.84	89.32	2.46	0.91
Seed production efficiency	310466638.86**	38423.81	1161147.27**	419447.58**	69442.9	2895.21	9.10	0.95
Economic growth rate	1926.35**	7.92	38227**	144.09**	53.89	41.75	17.58	0.83
Crude protein content (%)	23.81**	0.37	2.23**	1.98**	0.53	22.13	3.30	0.83

*** = Significant at p<0.001; ** = significant at p<0.01; * = significant at p<0.05; ns = no significance; CV = coefficient of variation.

Meenakshi et al., 2013) and mung bean (Singh et al., 2013). The primers were tested for their variability and reproducibility using four representative varieties selected based on their genetic background. Finally, 11 primers with good banding pattern, polymorphism and reproducibility for genotyping were used (Table 6).

ISSR-PCR amplification was carried out in a total reaction volume of 25 µl: 16.7 µl double-distilled water, 3.0 µl of $MgCl_2$ (25 mM), 2.5 µl of 10x PCR buffer, 1.0 µl of dNTP (100 mM equimolar solutions of each dATP, dCTP, dGTP and dTTP), 0.4 µl primer (20 mM), 0.4 µl (5 U/ µl) of Taq polymerase, and 1 µl of template DNA (70 ng/µl). PCR amplification were carried out in BiometraTpersonal (Applied Biosystems, USA). Amplification condition was set as: an initial denaturing at 94°C for 4 min followed by 40 cycles denaturation at 94°C for 15 s, primer-specific annealing temperature for 1 min and extension at 72°C for one and half min and completed with extension at 72°C for 7 min and the PCR products were stored at 4°C. The amplified products were separated on 1.67% agarose gel (w/v) in 1xTBE (Tris base, boric acid and EDTA) buffer and then post stained with ethidium bromide. The fragment size and molecular weight were estimated using 100 bp DNA ladder.

Data collection and analysis

Morphological data analysis

Field data were collected on either an individual plant basis for some characters (from five random plants) or on a plot basis according to descriptors of IBPGR, ICRISAT and ICARDA (1985) (Table 2). The protein analysis was measured based on Micro-Kjeldahl technique (AOAC, 2000). The SAS software packages (SAS Institute, 2003), was used to test the normality of the experimental error and detect the presence of outliers. Analysis of variance

(ANOVA) was performed using the generalized linear regression model to compare variation between the genotypes and means were separated by least significant difference (LSD) test at p<0.05. Genotypic and phenotypic variance with their coefficients of variation was estimated as described by Sharma (1998). Data for all traits were pre-standardized to means of zero and variance of unit before clustering and principal component analysis to avoid bias due to differences in measurement scales (Sneath and Sokal, 1973). Cluster analysis based on phenotypic traits using UPGMA clustering was done to group the faba bean genotypes into genetically distinct classes. Appropriate number of clusters was determined by using points where local peaks of pseudo F-statistics join with small values of the Pseudo t^2 statistics followed by a larger Pseudo t^2 for the next cluster fusion (SAS Institute, 2003). Genetic distance between pair of clusters as standardized Mahalanobis's D^2 statistics was calculated based on the recommendation of Singh and Chaudhary (1996). The principal component analysis (PCA) was done using MINTAB version 14.00 (MINTAB, 2003).

Molecular data analysis

Clearly distinguishable and reproducible fragments generated on gel from ISSR- PCR product was photographed using UV (Bioscens SC750) and scored based on a binary matrix "0" coded for absence, and "1" for presence of a band. Genetic diversity parameters: number of polymorphic loci, percent polymorphism, means of Nei's genetic diversity and Shannon diversity index were analyzed using POPGENE version1.32 (Yeh et al., 1999). Jaccard's similarity coefficients were computed using NTSYS- pc version 2.02 (Rohlf, 2000) set on SIMQUAL module. A dendrogram was constructed based on the similarity matrix using un-weighted pair group method with arithmetic mean procedure of the Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering methods (Sneath and Sokal, 1973). Principal coordinate analysis (PCoA) based on Jaccard's coefficient were done to examine the patterns of variation among individual genotypes using PAST version 1.18 (Hammer et al., 2001) software.

RESULTS AND DISCUSSION

Morphological diversity

Performance of varieties

The highest pooled mean yield was recorded for the variety, Walki (5391.84 kg ha^{-1}), whereas the lowest mean was obtained from Bako local variety (2955.59 kg ha^{-1}) (Table 1). The mean crude protein content over combined locations ranged from 20.59 to 22.92% for varieties Holetta-2 and Dagm, respectively. The report by Griffiths and Lawes (1978) revealed a wide range of crude protein contents variation among faba bean genotypes ranging from 20 to 40%; though, they observed low protein percentage from Ethiopian faba beans. Chavan et al. (1989) also reported variation among faba bean genotypes ranging from 20 to 41%, which agrees, at least in part, with the present study. Kelly (1973) and Bond et al. (1985) reported differences in the crude protein contents of legumes for environmental conditions, genotypes, and agricultural practices and reported inheritance of this trait is additive with some partial dominance.

Pooled analysis of variance across the three locations showed highly significant differences between varieties (*p<0.01*) for all measured traits (Table 2) consisted with observed genetic variability among the varieties. Genotype by environment interaction was highly significant (p< 0.01) for the traits considered except for plant height to first pod (p< 0.05). Sharifi (2015), Ammar et al. (2015) and Alghamdi (2007) reported highly significant differences in days to flowering and maturity, pod length, plant height, number of seeds per plant and thousand seeds weight. Sharifi (2014) found highly significant differences between faba bean genotypes from Iran in seed yield per plot, numbers of pods per plant, number of seeds per pod, pod length and thousand seeds weight. Gemechu et al. (2005) also reported highly significant differences between landraces across combined locations in days to maturity, thousand seeds weight and seed yield per plot. This indicates that the performance of faba bean varieties could be affected significantly by environmental condition. Ammar et al. (2015) reported similar result. This suggests that the performance of faba bean varieties is significantly and perhaps differentially affected by environmental conditions.

Estimation of variance components

The amount of genotypic and phenotypic variability existing in a species is the most important point for crop improvement selection criteria. High GCV (>10%) were observed for pod length, pod width, number of pods per plant, number of seeds per plant, biomass weight per plant, seed yield per plant, thousand seeds weight and seed production efficiency (Table 5). In contrast, the lowest GCV (<5%) were observed for leaf length, internode length, plant height, days to flowering, days to maturity and crude protein content. Higher genotypic coefficient of variation for thousand seeds weight and moderate for number of seeds per plant were observed for faba bean from Sudan (Aziz and Oman, 2015). They also reported low genotypic coefficient of variation for number of pods per plant, number of seeds per pod and seed yield per plant (Aziz and Oman, 2015). Tafere et al. (2013) also found high GCV for biomass and number of pods per plant in their study of genetic variability, heritability and correlation in some faba bean genotypes grown in Northwestern Ethiopia. Alghamdi (2007) also reported high genotypic coefficient of variation for days to flowering, number of pods plant and thousand seeds weight.

The highest PCV (>10%) were observed for traits such as leaf area, leaf number per plant, leaf area index, pod length, pod width, number of branches per plant, internode length, plant height to first pod, numbers of pods per plant, numbers of seeds per plant, biomass weight per plant, seed yield per plant, harvest index per plant, biomass weight per plot, seed yield per plot,

Table 3. Mean, genotypic variance, phenotypic variance, environmental variance, genotypic coefficient of variation, phenotypic coefficient of variation, heritability in broad-sense and genetic advance as percent in mean of Ethiopian faba bean.

Traits	Mean	σ^2e	σ^2g	σ^2gl	σ^2p	GCV (%)	PCV (%)	Hb (%)	GA	GA (%)
Leaf length (cm)	9.32	0.09	0.11	0.08	0.28	3.54	5.69	71.88	0.79	8.43
Leaf width W (cm)	4.49	0.08	0.09	0.03	0.20	6.59	9.99	78.30	0.72	16.11
Leaf area (cm^2)	29.72	10.21	7.83	1.10	19.14	9.41	14.72	79.09	7.13	23.99
Leaf area index	223.20	1326.59	219.63	870.33	2416.55	6.64	22.02	30.05	30.43	13.63
Pod length (cm)	7.28	0.25	1.00	0.03	1.27	13.71	15.49	95-.20	2.21	30.37
Pod width (cm)	1.34	0.03	0.02	0.00	0.05	11.28	17.22	81.03	0.38	28.75
Internod length (cm)	5.93	0.30	0.04	0.11	0.44	3.21	11.20	29.81	0.41	6.88
Plant height to first pod (cm)	43.79	22.78	13.79	6.52	43.09	8.48	14.99	69.78	9.44	21.55
Plant height (cm)	149.07	15.56	20.18	13.28	49.02	3.01	4.70	74.19	10.70	7.18
Numbers of branch per plant	1.69	1.20	0.02	0.12	1.34	8.81	68.57	8.45	0.20	11.94
Number of pod per plant	33.82	51.61	33.62	0.77	86.00	17.14	27.42	79.15	15.12	44.71
Number of seed per plant	88.43	284.56	203.13	22.19	509.88	16.12	25.54	78.75	36.63	41.42
Number of seed per pod	2.63	0.02	0.01	0.02	0.05	4.34	8.25	60.86	0.27	10.34
Biomass per plot (g/plot)	3361.72	90807.71	104172.55	71444.90	266425.15	9.60	15.35	72.79	773.93	23.02
Seed yield per plot (g/plot)	1438.22	10620.27	15078.15	39120.96	64819.38	8.54	17.70	50.45	264.58	18.40
Harvest index (%)	43.39	44.90	9.16	8.83	62.89	6.98	18.28	46.78	7.64	17.61
Thousand seed weight (g)	489.88	4873.54	4854.86	6334.47	16062.86	14.22	25.87	62.41	162.95	33.26
Days to flowering	54.44	1.40	0.68	1.83	3.90	1.51	3.63	44.49	1.81	3.33
Days to flowering	143.77	4.21	4.49	14.87	23.58	1.47	3.38	44.26	4.43	3.08
Seed filling period	89.33	4.84	5.42	14.04	24.30	2.61	5.52	49.70	5.05	5.65
Seed production efficiency	2895.21	69442.90	123619.45	174993.84	368056.19	12.14	20.95	63.88	798.32	27.57
Economic growth rate	41.75	53.89	39.70	45.10	138.68	15.09	28.20	62.31	15.12	36.20
Crude protein content (%)	22.13	0.53	0.04	0.73	1.30	0.92	5.15	11.23	0.26	1.19

σ^2e = Environmental variance; σ^2g = genotypic variance; σ^2gl = genotype by environment interaction variance; σ^2p = phenotypic variance; GCV = genotypic coefficient of variation; PCV = phenotypic coefficient of variation; Hb = heritability; GA = genetic advance.

harvest index, thousand seed weight, seed production efficiency and economic growth rate (Table 3). In this study, the PCV values were greater than GCV values across the environment which is consistent with other scientist report (Alghamdi, 2007; Tafere et al., 2013; Aziz and Oman, 2015).

Heritability and genetic advance as a percent of mean

In this study, heritability value ranged from 8.45 to 95.2% for number of branches per plant and pod length, respectively (Table 3). Heritability values were sufficiently high for traits like pod length (95.2%) and pod width (81.03%) suggesting that these traits could be selected for in tradition breeding program. This show that environmental factors exerted minimal influence on detectable heritability, that is, environmental responses were separate heritable component (Sharifi, 2015). It is concluded that selection based on means would be useful for the selection for these traits in the faba bean varietal development.

Moderately high heritability values were observed for characters such as leaf length, leaf width, leaf area, plant height to first pods, plant height, number of pod per plant, number of seed per plant, number of seed per pod, biomass weight per plot, thousand seed weight, and seed production efficiency which indicates the possibility of improvement via selection for these traits. Consistent with the results presented here, other workers have reported moderately high heritability for these traits. Hence, high heritability values for most of the characters could be

Table 4. Lists of genotypes grouped under different clusters.

Clusters	Number of genotypes	Name of varieties
1	10	Mosisa, Adet Hanna, Tesfa, Mesay, NC-58, Degaga, Kassa, Kuse, Bulga 70, Sinana Local
2	14	Tumsa, Gebelcho, Holetta-2, Hachalu, Obse, Gachena, Didae, Shallo, Walki, Moti, EKCSR-02006, Agarfa local, Adet local, Kulumsa local
3	6	Dagm, Wayu, CS-20-DK, Lalo, Bako local, Eniwari local
4	1	Dosha
5	1	Gora

attributed to the relatively favorable environment at combined locations (Alghamdi, 2007; Mellion et al., 2012; Teferen et al., 2013; Aziz and Oman, 2015). Intermediate heritability values were recorded for harvest index, grain yield, grain filling period, days to flowering, days to maturity, biomass weight per plant and seed yield per plant. Alghamdi (2007) obtained the highest heritability for days to flowering and maturity. Conversely, low heritability values were recorded for leaf number per plant, leaf area index, internode length, number of branch per plant, harvest index per plant and crude protein content.

Genetic advance as a percent mean ranged from 1.19% for crude protein content to 44.71% for number of pods per plant (Table 3). Johnson et al. (1955) concluded that broad sense heritability, together with genetic advances are usually more useful than heritability alone in predicting the resultant effect of selection. In the present study, high genetic advances as percent of mean with high heritability was observed on traits: pod length, pod width, leaf area, number of pods per plant, number of seed per plant, biomass weight per plot, thousand seed weight and seed production efficiency. The report by Kalia and Sood (2004) showed high heritability and high genetic advance for number of pod per plant which indicated high additive gene action and possibility of trait improvement through selection.

Extent and pattern of diversity based morphological characteristics

Cluster analysis based on morpho-agronomic traits distinguished five distinct groups of faba bean genotypes. The number of individuals in each of the five clusters ranged from one to fourteen in the smallest and largest clusters, respectively. Cluster I consisted of 9 genotypes, cluster II of 14 genotypes, cluster III of 6 genotypes, cluster IV and V, one genotype each (Table 4 and Figure 1). The first cluster contained genotypes derived from hybridization, introduced materials and selected from landraces. Even though genotypes were grouped together based on their morphological similarity, the clusters did not necessarily include all genotypes from same genetic background. The genetic diversity of faba bean genotypes using cluster analysis and relationships within and among individuals and populations has been described elsewhere (Polignano et al., 1993; Gemechu et al., 2005; Chaieb et al., 2011; Yahia et al., 2012).

The pair wise generalized squared distances (D^2) showed highly significant difference ($p<0.01$) among inter-clusters (Table 5). The maximum distance was found between C1 and C5 (D^2 =554.60), while a minimum distance (D^2 = 54.80) was observed between C1 and C3. The high values of inter cluster distances indicated divergence among the varieties and might be used in breeding programs for better genetic recombination and selection of genetically divergent parents for exploitation in crossing programs. This finding is consistent with Million and Habtamu (2012) who used twenty-five elite faba bean genotypes to study genetic variability of seed yield and yield related traits and found high D^2 value. Gemechu et al. (2007) also reported divergence between Ethiopian germplasms.

The principal components analysis (PCAs) with Eigenvalue greater than one contributed 85% of the entire diversity among the genotypes. The first three PC_s contributed 66% (PC1 = 38%, PC2 = 19% and PC3 = 9%) of total variation among Ethiopian faba bean varieties. This agrees with the results reported by Gemechu et al. (2005) and Yahia et al. (2012).

Molecular diversity

Magnitude of diversity as revealed by ISSR markers

Eleven ISSR primers amplified a total 120 bands, of which 107 loci were polymorphic ranging from 5 (ISSR 818) to 17 (ISSR 811) with an average of 90% polymorphism (Table 6). The size of all amplified bands ranged from 200 to 3000 bp (Figure 2). Average number of bands and polymorphic fragments per primer were 11 and 10, respectively. Analysis of percent polymorphisms per primer signified 100% polymorphic for primers ISSR 811, ISSR 860, ISSR 873 and ISSR 881 followed by the primer ISSR 854 with 90% polymorphisms, indicating that these primers were much better for resolving genetic

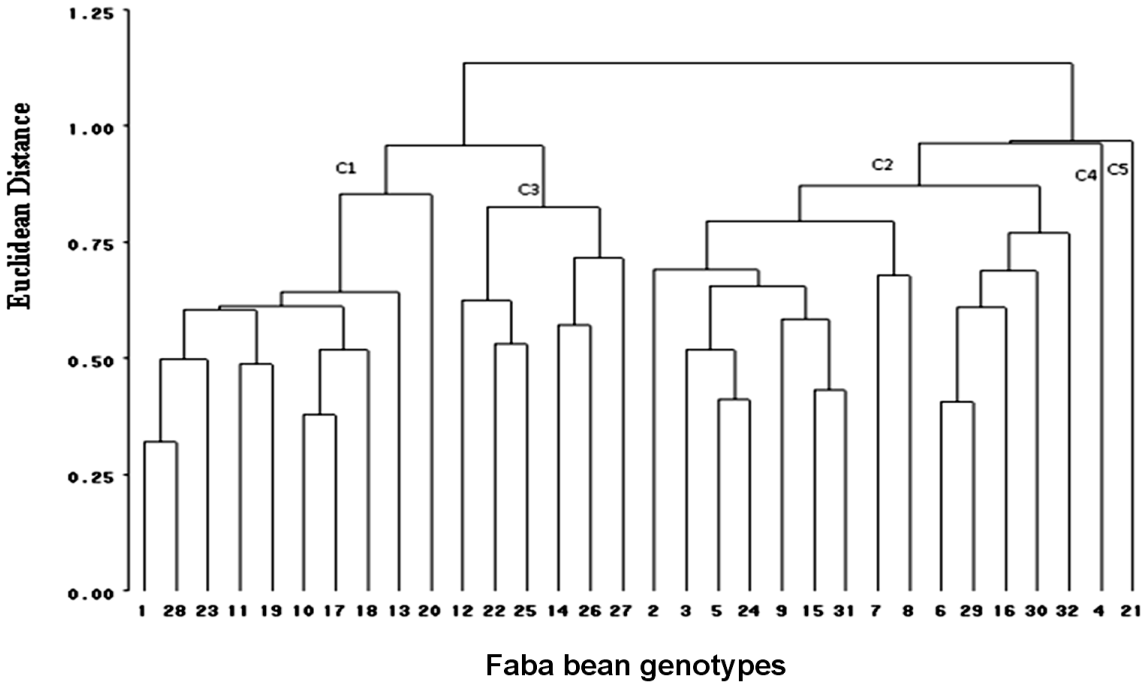

Figure 1. Dendrogram with UPGM and Euclidean distance showing relationship among 32 Ethiopian faba bean genotypes using 23 quantitative traits. Where 1 = Mosisa; 2 = Tumsa; 3 = Hachalu; 4 = Dosha; 5 = Gachena; 6 = Walki; 7 = Obse; 8 = Moti; 9 = Gabelcho; 10 = Adet Hanna; 11 = NC-58; 12 = Wayu; 13 = Degaga; 14 = Dagm;15 = Holetta-2; 16 = Shalo; 17 = Tesfa; 18 = Mesay; 19 = Bulga 70; 20 = Kassa; 21 = Gora; 22 = CS-20-DK; 23 = Kuse; 24 = Dida'a; 25 = Lalo; 26 = Bako local; 27 = Eniwari local; 28 = Sinana local; 29 = Agarfa local; 30 = EKCSR-02006; 31 = Adet local; 32 = Kulumsa local.

Table 5. Distances between nine clusters of 32 faba bean varieties grown in Ethiopia.

Clusters	C1	C2	C3	C4	C5
C1	0	80.54**	54.80**	411.55**	554.60**
C2		0	89.76**	376.91**	358.07**
C3			0	388.79**	459.37**
C4				0	306.27**
C5					0

** = Highly significant at probability level, $p<0.01$ ($\chi^2_{22} = 40.29$).

diversity of faba bean varieties

There is substantial variation in the degree of polymorphisms in reported genetic diversity of faba bean germplams using ISSR markers in the literature, while investigators have reported lower average percentages polymorphism, than found in the present study on genetic diversity of faba bean germplasms using ISSR markers (Terzopoulos and Bebeli, 2008; Abdel-razzak et al., 2012; Basheer-Salimia et al., 2013; Hou et al., 2014; Aziz and Oman, 2015).

Recently, Ammar et al. (2015) showed highest level of percent polymorphisms (100%) using 6 SRAP and 4 AFLP primers on faba bean genetic diversity assessment from Saudi Arabia. Mejri et al. (2012) also described 97.3% of polymorphism to study the effect of gamma radiation on 22 faba bean genotypes from Tunisia using15 ISSR primers. Similarly, Wang et al. *(*2012) reported percentage of polymorphisms that ranges from 91 to 100% with an average of 93% in a study of genetic diversity and relationship of global faba bean germplasms collected from across the world. Salazar-Laureles et al. (2015) also found percent polymorphisms ranging from 71.4 to 100% with an average of 91.3% in an analysis of genetic variability within Chilean faba bean accession using ISSR markers.

The higher average percent polymorphism per primer

Table 6. Diversity parameters used to reveal diversity of Ethiopian faba bean varieties based on 11 ISSR primers

Primer	RM	NSB	NPL	PP	Na	ne	h	I
811	(GA)8C	17	17	100.00	2.00±0.00	1.40±0.31	0.25±0.15	0.41±0.18
812	(GA)8G	15	12	80.00	1.800±0.41	1.45±0.40	0.26±020	0.39±0.28
818	(CA)8G	7	5	71.43	1.71± 0.49	1.38± 0.40	0.23±0.20	0.35±0.28
848	(CA)8RG	9	7	77.78	1.78±0.44	1.27±0.27	0.18±0.15	0.30±0.22
854	(TC)8RG	10	9	90.00	1.90±0.32	1.32±0.2	0.22±0.12	0.37±0.18
857	(AC)8YG	10	8	80.00	1.80±0.42	1.30±0.37	0.18±0.20	0.28±0.27
860	(TG)8RA	9	9	100.00	2.00±0.00	1.66±0.29	0.38±0.11	0.56±0.13
864	(ATG)6	11	9	81.82	1.82±0.40	1.36±0.32	0.23±0.18	0.36±0.24
873	(GACA)4	13	13	100.00	2.00±0.00	1.57±0.34	0.33±0.15	0.50±0.18
880	(GGAGA)3	9	8	60.00	1.60±0.52	1.29±0.36	0.18±0.20	0.27±0.28
881	(GGGTG)3	10	10	100.00	2.00±0.00	1.52±0.31	0.32±0.13	0.49±0.16
Total		120	107	90.00	1.90±0.30	1.43±0.32	0.27±0.16	0.41±0.23

RM = repeat motif; NSB = numbers of scored bands; NPL = number of polymorphic loci; PP = percent polymorphisms; Na = number of allele; ne = effective number of allele; h = gene diversity; I = Shannon diversity index.

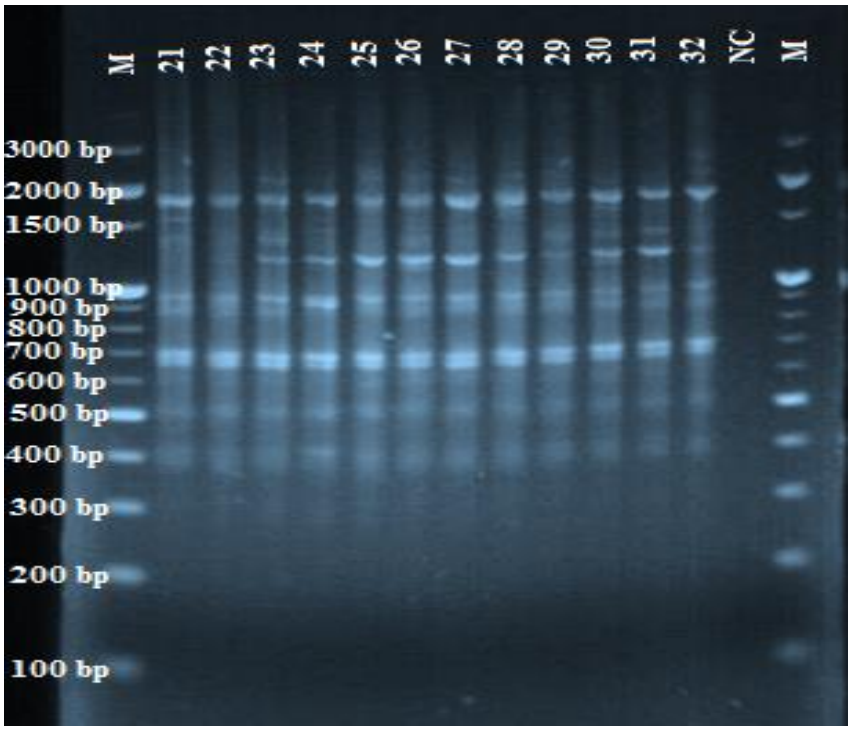

Figure 2. ISSR fingerprint generated from 12 individuals of faba bean varieties using primer 873.

(90%) observed in this study showed that the capacity of ISSR primers were capable of revealing the genetic diversity within and between groups of faba bean varieties, at least those investigated here. The ISSR primers considered in the present study could be used in further studies to identify genetic diversity of faba bean germplasms. The degree of polymorphism among the groups/category of faba bean varieties ranged from 37.5% (45 loci) for the local (farmer) varieties to 84.17% (101loci) for the varieties derived from hybridization (Table 7). The highest polymorphisms observed in the varieties derived from hybridization as compared to local varieties could also be explained by the broader spectrum initially acquired from subsequent genetic recombination during crossing program in the national breeding programs which introduced new alleles to these

Table 7. Diversity parameters indicating the variability of four categories of faba bean genotypes in the present study.

Population type	NPL	PP	na	ne	h	I
Hybridization	101	84.17	1.84+0.37	1.44+0.34	0.27+0.17	0.40+24
Local selection	92	76.67	1.77+0.42	1.39+0.34	0.24+0.18	0.37+0.25
Introduction selection	56	44.67	1.44+0.5	1.30+0.35	0.17+0.20	0.26+0.28
Local varieties	45	37.5	1.37+0.48	1.19+0.32	0.12+0.17	0.18+0.25
Over all genetic diversity	107	90.00	1.90±0.30	1.43±0.34	0.27±0.16	0.41±0.23

NPL = number of polymorphic loci; PP = percent polymorphisms; na = number of allele; ne = effective number of allele; h = gene diversity; I = Shannon diversity index.

varieties.

Using 11 ISSR primers, varieties developed via hybridization revealed the highest gene diversity (h = 0.27) and Shannon diversity index (I = 0.41), whereas farmer (local) varieties showed the least gene diversity (0.12) and Shannon diversity index (0.18). The average gene diversity and Shannon diversity index per primer ranged from 0.18 to 0.38 and 0.27 to 0.56 with mean value of 0.27 and 0.41, respectively (Table 6). The present study is comparable with Wang et al. (2012) who used 11 ISSR primers and found gene diversity and Shannon diversity indices ranging from 0.18 to 0.26 and 0.27 to 0.39, respectively using 11 ISSR primers.

The highest gene diversity (0.38) and Shannon index (0.56) were recorded by primer 860 and followed by primer 873 with high gene diversity (0.33) and Shannon index (0.50) and this indicated that primers 860 and 873 were better able to detect genetic diversity of these Ethiopian faba bean varieties. The least gene diversity (0.18) was obtained from primers 848 and 857.

Cluster analysis and pattern of grouping

UPGMA cluster analysis based on data from 11 ISSR primers grouped faba bean varieties into three distinct clusters and showed relationships among Ethiopian faba bean varieties (Figure 3). However, some of the varieties spread all over the dendrogram without forming strict grouping based on their breeding information. The out-crossing habit of faba bean has its own impact on the intermixing of varieties from different genetic backgrounds into similar or the same cluster. The C1 mostly contained varieties derived from hybridization which includes: Mosisa, Hachalu, Tumsa, Gachena, Obse, Walki, Moti and Gebelcho and two varieties derived from local collection (Dosha and Adet Hanna). The other possible reasons for grouping of these varieties into same cluster could be the breeding objectives designed by breeder. The breeding objectives of faba bean were ultimately designed to improve faba bean genotypes for their seed yield, resistance to biotic and abiotic factors and recently for seed size. Therefore, these common objectives could make the materials to carry similar gene responsible for yield, seed size and resistance to biophysical stresses

The present clustering concedes with the finding of Abdel-razzak et al. (2012) who studied the genetic diversity in 10 Egyptian faba bean genotypes using ISSR grouped into individuals depending on their genetic similarity. Wang et al. (2012) also grouped 802 global faba bean accessions into four groups based on their genetic similarity in their studies of genetic diversity and relationship of global faba bean accession. Salazar-Laureles et al. (2015) grouped 39 faba bean accessions into six clusters based on their genetic similarity coefficient ranging from 0.38 to 0.83, suggesting wide genetic variability between accession at molecular level. So, these findings agree with the recent result obtained in genetic diversity studies of Ethiopian faba bean varieties.

From the cluster analysis, the estimated genetic similarity among faba bean varieties in this study ranged from 0.43 to 0.77 (Figure 3). Depending on an estimated genetic similarity matrix, the highest genetic similarity value was observed between Mesay and Bulga-70 (0.77), followed by between Sinana local and Agarfa local (0.76) and between Kuse and Lalo (0.75). The causes of high similarity between Sinana and Agarfa local varieties, could be, both varieties are found in similar geographical location and the probability of seed exchange between farmers is high. Kuse and Lalo varieties also showed high genetic similarity with each other and these varieties were released for vertisol areas and they could carry similar gene to tolerate waterlogging problem. This similarity coefficient shows that these varieties are genetically more similar and hence the hybridization between these groups may not be considered useful in getting desirable segregating materials. The least similarity value was observed between varieties Obse and Didea (0.29), followed by association between Gachena and Didea (0.32), Tumsa and Didea (0.35) and Mosisa and Lalo (0.36), which were the most genetically distant of all varieties in the present study. It is suggested that it may be useful to include such lines in hybridization programs that seek to enhance genetic variability of Ethiopian faba bean varieties. This may additionally

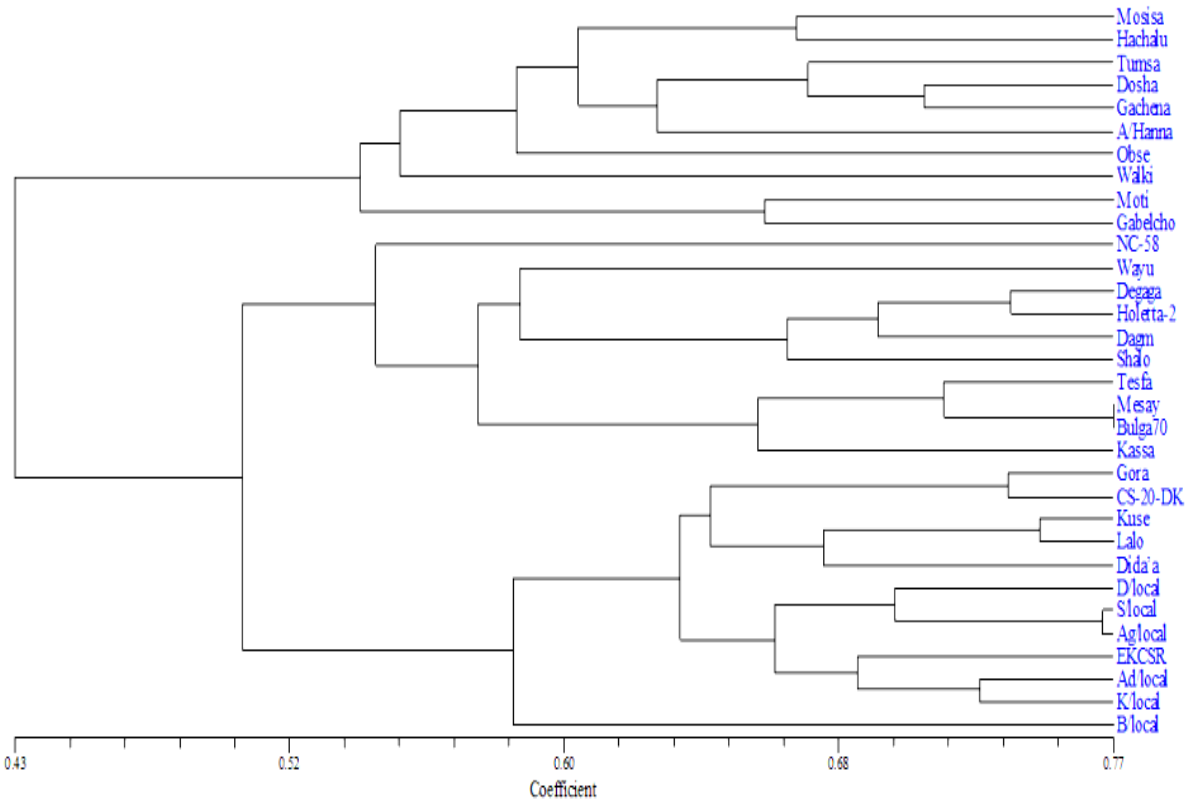

Figure 3. Dendrogram for 32 faba bean genotypes obtained using UPGMA of 120 amplified bands by 11 ISSR primers. The UPGMA algorithm is based on Jaccard's similarity coefficients obtained after pair wise comparison of the presence-absence fragments.

provide insight into the potential for trait selection from F2 and subsequent segregating generations from hybridization programs.

Principal coordinates analysis (PCO) based on ISSR data clearly differentiated Ethiopian faba bean varieties and resolved these varieties into three distinct groups. The first three groups predominantly separated faba bean varieties on the basis of their pedigree relationships and , when taken together, explained 24.02% of total variation with PC_1, PC_2 and PC_3 explaining 11.48, 7.28 and 5.26%, respectively. This result is consistent with other studies on faba bean genetic diversity study using ISSR markers (Wang et al., 2012; Salazar-Laureles et al., 2015). Local varieties were clearly separated from the cultivars by dendrogram and PCoA clustering. The "Farmer" variety from Bako, clustered separately from the groups in both dendrograms. Bako and Kulumsa local varieties separated solely from the group in PCoA and indicated genetic distinctness from the materials used in the present study.

Conclusions

Generally, the presence of genetic diversity within a given breeding population provides primary resources of potentially used traits. Methods of identification of useful heritable traits could play an essential role in designing better breeding strategies for genetic improvement aimed at solving the needs of the producers. For such reasons, genetic diversity is a resource that can contribute to the well-being of the present and future generations, if useful traits can be identified and incorporated into gerplasms. This study revealed considerable amount of genetic variation between cultivated faba bean varieties in Ethiopian. However, some varieties were quite more similar which points to the need to broaden the genetic base. Moreover, there should be efforts to maintain and improve gene pool of released faba bean cultivars of Ethiopia by involving divergent parents in the crossing program. The observed genetic variation showed an opportunity of using these materials in a future faba bean breeding program via introgression with other germplasm resources (from either introduction or landrace materials).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Molecular characterization and tissue expression analysis of five genes for chitinase in the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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Insect chitinases are hydrolytic enzymes that cleave chitin of the cuticle and peritrophic membrane during molting. Multiple genes encode insect chitinases, which are characterized as having diverse chemical and enzymatic properties depending on the time and the site of expression. This work was done to isolate and characterize chitinase genes from the red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliver), a cryptic pest of many palm trees. The isolated five genes were phylogenetically clustered into five different groups (I, II, III, VI, and VII) of the glycoside hydrolase family 18 (GH18). Domain structure analysis revealed that *RfCht1* (group I), *RfCht3* (group VI), and *RfCht5* (group VII) each retained a single catalytic domain of the GH18, whereas *RfCht2* (group III)) and *RfCht4* (group II) possessed two and five GH18 catalytic domains, respectively. *RfCht1*, *RfCht2*, and *RfCht3* each retained a single chitin-binding domain (CBD) and *Rf*Cht4 retained five CBDs, but *RfCht5* lacked CBD. Developmental and tissue expression profiles showed high levels of transcripts of the five genes in the newly hatched first instar larvae. *RfCht1* and *RfCht2* transcripts were expressed constitutively almost with high levels in young and mature eggs, in all tested larval instars, pre-pupae, pharate pupae, and adults; whereas *RfCht3* and *RfCht5* transcripts were expressed as low levels in the early instars larvae few hours before molting. In late developmental stages and mature eggs, *RfCht3*, *RfCht4*, and *RfCht5* were expressed as low levels mainly in the cuticle. This study presents the first report on chitinase genes in the RPW and suggests that these genes have additional roles in the weevil development, which require further elucidation.

Key words: Chitinase, conserved motifs, cuticle, domain structure, expression profile, *Rhynchophorus ferrugineus*

INTRODUCTION

Insect body contains rigid and insoluble chitin, as a component of the exoskeleton, trachea, and the peritrophic membrane (PM) that surrounds the food in the midgut. Chitin in these organs provides protection to

insects against environmental and mechanical injuries but it limits the growth and development of insects. Therefore, the cuticles and PM are degraded periodically and reshuffled to allow growth and development (Merzendorfer and Zimoch, 2003; Muthukrishnan et al., 2016). Insect chitinases play crucial roles to degrade chitin in the old cuticles and PM during the larval molting and pupation and act defensively to prevent bacteria and fungi from penetrating the PM. They belong to family 18 glycosyl hydrolases (GH18). The GH18 genes of chitinases have potential use for pest management as biopesticides (Kramer and Muthukrishnan, 1997). Insect chitinase genes have been suggested as targets for gene silencing via RNA interference (RNAi) (Al-Ayedh et al., 2016; Cao et al., 2017; Su et al., 2016; Zhu et al., 2008a) and have also been proposed as appropriate candidates in host-mediated silencing of pest genes (HMSPG) for control of diseases and insect pests of date palm (Niblett and Bailey, 2012).

Chitinases have been isolated and characterized in many insects including *Anopheles gambiae*, *Bombyx mori*, *Chilo suppressalis*, *Drosophila melanogaster*, *Manduca sexta*, *Mythimna separata*, *Nilaparvata lugens*, *Ostrinia nubilalis*, *Tribolium castaneum*, and economically important other species (Shen and Jacobs-Lorena, 1997; Zhu et al., 2008b; Khajuria et al., 2010; Zhang et al., 2011a; Huang et al., 2012; Pan et al., 2012; Tetreau et al., 2015; Xi et al., 2015; Su et al., 2016; Cao et al., 2017). Functional analyses of particular chitinases revealed that insect chitinases belong to a large family of enzymes with diverse domain architecture, expression patterns, tissue specificity, and function. They have been grouped into, at least, eleven groups based on domain arrangement and/or tissue specificity of expression and phylogenetic analyses as well as functional analyses (Nakabachi et al., 2010; Tetreau et al., 2015). Groups I and II chitinases are found in molting fluid (Koga et al., 1992; Qu et al., 2014). Groups III and VIII members have a membrane-spanning domain and are involved in regulating abdominal contraction and wing expansion. Group V members are mainly imaginal disc growth factor genes that are necessary for adult eclosion (Zhu et al., 2008a). It has been reported that in *N. lugens* at least four groups of chitinases (groups I, II, III, and V) are involved in nymph-nymph molt (Xi et al., 2015). The presence of several functional chitinases with distinct domain configuration suggests that they have other functions besides the digestion of chitin in the old cuticle. These could include providing primers for elongation of chitin and processing of mature chitin chains for a higher level of organization (Muthukrishnan et al., 2016).

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a noxious internal feeder attacking many palm species including coconut, *Cocos nucifera*, date palm, *Phoenix dactylifera,* Canary Islands palm, *Phoenix canariensis*, and African oil palm, *Elaeis guineensis* (Dembilio et al., 2012; Fiaboe et al., 2012; Hoddle et al., 2013). The entire larval life cycle of the weevil is concealed within the palm trunk, which makes its early infestations difficult to detect (Faleiro et al., 2012; Hoddle et al., 2013; Llácer et al., 2010; Murphy and Briscoe, 1999). Food and Agriculture Organization of the United Nations (FAO) has classified RPW as category-1 pest on date palm in the Middle East (Al-Dosary et al., 2016).

To date, the large-scale dataset on the RPW transcriptome analyses though provided substantial information on the weevil's development that could have important practical applications (Wang et al., 2013); however, no information exists about particular genes, especially those functions relating to processes of ecdysis and metamorphosis of the developmental stages of the RPW. Thus, we isolated, amplified, cloned, and sequenced five genes for chitinase were isolated from the RPW to gain insight into their structural and functional domains architecture and also to study the mRNA expression patterns of these genes in different developmental stages of the RPW.

MATERIALS AND METHODS

RPW rearing and tissue collection

RPW was reared in the laboratory and the insectary facilities of the Date Palm Research Center of Excellence, King Faisal University. For egg laying, male and female adults were fed on sugarcane kept in TATAY storage boxes (51 cm × 38 cm × 26 cm) made of polypropylene and bisphenol A (BPA) free (www.tatay.com) with perforated lids as described elsewhere (El-Shafie et al., 2013). Eggs were removed with a brush and placed into Petri dishes that contained cotton and moist filter paper and incubated at 28°C until the eggs hatch. First instar larvae were collected daily and reared on pineapples and date palm trunk. Samples of different developmental stages were collected periodically for integument and tissue collection. Larvae were dissected by cutting off their heads using a standard stainless steel entomology dissection set. The integument was cut longitudinally to separate the adipose tissues and the guts. The dissected tissues were immediately frozen in liquid nitrogen. Eggs, elytra, forewings, and the adult's body were directly frozen in liquid nitrogen. All samples were stored at -80°C for the subsequent experiments.

BLAST® search and sequence alignment

The online Basic Local Alignment Search Tool (BLAST) was used to search for potential chitinase genes sequences in the RPW Transcriptome Shotgun Assembly (TSA) (Wang et al., 2013). *T. castaneum* and *B. mori* chitinase gene sequences available in the

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Table 1. Primer sequences for amplification and expression profiling of the RPW chitinase genes (*RfChts*).

Primer name	Sequence (5′→ 3′)	Target Gene	Purpose
RfCht1-1	GGACACTCGGTTGTGGTGCTTTTGGTGG	*RfCht1*	Full length cDNA cloning and qPCR
RfCht1-5c	TTCGGAAACAAAATTGATTTATTTTCGT		Full length cDNA cloning
RfCht1-2	CCTGGGATATTCTCATCATTGATCCTGA		RT-PCR
RfCht1-13c	CAGCCCAGTTTCCACGCAAATCGTACGT		RT-PCR
RfCht2-1	GCCAAGGACGTCGACTGGGCTGTCGTGG	*RfCht2*	Full length cDNA cloning
RfCht2-4c	GCGATGTTAATATATTTTTATTATAAGA		Full length cDNA cloning
RfCht2-7	CATCCGACAACGCAGTGAGCGCCTCCAG		qPCR
RfCht2-5c	TGCATTTGATGGAGAGTGTACGGTATATTTTG		qPCR
RfCht2-9c	GTCGGGACCTACTGGAACAGCAGCTGAG		qPCR
RfCht3-1	GAATTAAGTCTTCGTACCGTACGCTTAG	*RfCht3*	Full length cDNA cloning
RfCht3-3c	CGGTGAGGATGGGGTTGTTGTAACCACT		Full length cDNA cloning and qPCR
RfCht3-3	GGATAACTGCAGCTACTAGCAAATCTAG		qPCR
RfCht3-6c	CGTTGATGGTCGTTGACGCCTGATATTG		qPCR
RfCht4-1	CGTTACTTGGCGACATCGGCATCGACGG	*RfCht4*	Full length cDNA cloning
RfCht4-6c	AGACGTTATCGGTGTATTCTTTTTGACA		Full length cDNA cloning
RfCht4-2	GGTGGAACTCGCCAGGATGCTCCCCATC		qPCR
RfCht4-2c	CGCCCAGTTGGTTACGTAGCACACCACC		qPCR
RfCht4-12	GCGTATTCGCATGGGCTGCAGATCTGGA		qPCR
RfCht4-14c	GCTGATCCGTCGCTGACCAATCTAGAGT		qPCR
RfCht5-1	TACCAGTTAGAACGGTCGAGCTTCGACC	*RfCht5*	Full length cDNA cloning
RfCht5-2c	AAATCCCATAAGGAACAATATAGTATAAAATA		Full length cDNA cloning and qPCR
RfCht5-2	CCCCTGACAAGCACCGACGTCCTCAGCG		qPCR
RfCht5-1c	CGCTGAGGACGTCGGTGCTTGTCAGGGG		qPCR
RfCht5-3	CATAGGCGGTTGGAACGAGGGTTCCACC		qPCR

GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used to search for similar sequences in the RPW TSA dataset. The identified RPW TSA sequences were pools of unannotated sequences with gaps in sequenced contigs. Multiple sequence alignment was done using MEGA7 (Kumar et al., 2016) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) softwares in order to locate the four highly conserved signatures in the amino acids of all known insect chitinases (Zhang et al., 2011b). Only RPW TSA contigs with the conserved four regions were used to synthesize the primers (Table 1) used for partial amplification of chitinase genes.

RNA isolation and first strand cDNA synthesis

Frozen RPW tissues were ground into fine powder in liquid nitrogen using mortar and pestle. Total RNA was isolated using RNeasy Plus Universal Mini Kit (QIAGEN) according to the manufacturer's protocol. Elongase™ enzyme mix was obtained from Invitrogen® and the recombinant Taq DNA polymerase was purchased from Fermentas®. Reverse transcription of RNA to synthesize first strand cDNAs for *RfChts* was done using a random hexamer primer and RevertAid RT Kit obtained from Thermo Fisher Scientific according to the manufacturer's protocol. Double-stranded cDNA was amplified using the first strand cDNA as template and a gene-specific primer (Table 1). Primers used to amplify the full-length cDNAs and to study the expression patterns of the RPW chitinase genes, and for sequencing are shown in Table 1. The thermocycler used for cDNA amplification was Veriti® Thermal Cycler (96 well) supplied by Applied Biosystems™. Amplified PCR products were electrophoresed on 0.7% agarose D1 (Pronadisa) gel, stained with ethidium bromide, visualized using INGENIUS Syngene Bio Imaging System, and documented using GeneSnap software from Syngene. Then, the cDNAs were purified either from the excised gel using QIAquick® Gel extraction kit (Qiagen) or directly from the PCR products using the DNA Pure Kit (Geneaid®) following the manufacturers' protocols. The recovered cDNAs were used for the subsequent PCR amplification, cloning, or direct sequencing.

Gene cloning and sequencing

The PCR-amplified cDNAs were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Ligation, cloning, and transformation processes were carried out according to the standard protocols (Sambrook et al., 1989). The manipulated plasmids were transformed into *Escherichia coli* strain DH5α. Plasmids maintained by the bacterium were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the supplier's instructions. Due to the big sizes of some cDNA clones, multiple

Table 2. Properties of the identified red palm weevil, *Rhynchophorus ferrugineus*, chitinases (*RfChts*).

Gene name	Group	cDNA length (bp)	Coding region (bp)	5′-untranslated region	3′-untranslated region	Amino acids	Isoelectric point	Molecular weight (kDa)	GenBank accession #
RfCht1	I	1,828	103~1,722	102	106	540	5.43	60.70	KX954127
RfCht2	III	3,638	68~3,031	67	607	988	6.64	112.49	KY576032
RfCht3	VI	4,124	169~4,083	168	41	1305	5.33	145.53	KY576033
RfCht4	II	9,058	209~8,746	208	312	2846	6.53	319.97	KY576034
RfCht5	VII	1,595	61~1,404	60	191	448	6.73	50.74	KY576035

sequencing rounds were carried out to clarify dubious and long uncovered reads. Sequencing was done at Macrogen service facilities (Seoul, South Korea).

Domain structure and phylogenetic analyses

Multiple sequence alignment of the deduced amino acids of *RfChts* and the molecular phylogenetic analyses were performed using the software MEGA7 (Kumar et al., 2016). Chitinase domain structure analysis was done using InterPro: protein sequence analysis and classification database (https://www.ebi.ac.uk/interpro/).

Expression profiles of *RfChts* genes in different developmental stages and tissues

The expression profiles of the five *RfCht* genes at different stages of development including eggs, larvae, pre-pupae, pupae, adults and appendages were tested. Eggs were collected 12- and 24-h after laying and tissues of the middle-aged larvae were collected at 0- to 96-h pre-molting. Reverse transcription PCR was done in a 25-µl reaction mixture containing 1 µl template first strand cDNA, 10 pmol/µl each primer, 12.5 µl Master Mix (Biomatik Corporation, Canada), and nuclease-free water. The thermocycler program for RT-PCR was as follows: initial denaturation cycle at 94°C for 3 min followed by 30 cycles at 94°C for 25 s, 60°C for 25 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gel. Experiments were replicated at least three times using independent total RNA preparations. RPW's ribosomal protein S3 (*RfRpS3*) was used as an internal reference gene for RT-PCR analysis.

RESULTS

Sequence analysis of cDNAs for the genes of chitinase

Five chitinase cDNA amplicons from *R. ferrugineus* were sequenced (*RfCht1*, *RhCht2*, *RfCht3*, *RfCht4*, and *RfCht5*) and the sequences were deposited in the GenBank database and the accession numbers are shown in Table 2. The *RfCht1* full sequence is 1,828 bp long. It consists of 1620 bp open reading frame (ORF), 102 bp 3′-untranslated region, and 106 bp 5′-untranslated region (Table 2). The translated region consists of 540 amino acids (Figure 1). The first 20 amino acids constitute a putative signal peptide, as predicted by the online SignalP 4.1 Server (Petersen et al., 2011) that targets the protein to the extracellular space or sorts it into plasma membrane to face in both cases carbohydrates of the extracellular matrix (Kawamura et al., 1999; Royer et al., 2002; Arakane et al., 2003).

Analysis of the putative consensus signature domains of the isolated cDNA revealed that the protein consists of a single catalytic domain of the glycoside hydrolase family 18 (GH18). The catalytic domain covers the deduced amino acids span from 18 to 383. The amino acids from 270 to 346 form a chitinase insertion domain (CID). The C-terminus amino acids from 484 to 539 constitute a chitin-binding domain (CBD), which belongs to family 14 of carbohydrate-binding modules (CBM14). *RfCht2* is 3,638 bp long covering an ORF of 2,964 bp that encodes a putative protein of 988 amino acids including a putative signal peptide, two GH18 catalytic domains in which two CIDs are embedded, and has a single C-terminal CBD. *RfCht3* is 4,124 bp long. It consists of 3,915 bp ORF that encodes a putative protein of 1,305 amino acids including a putative signal peptide, a GH18 catalytic domain in which a CID is embedded, and has a single C-terminal CBD. On the other hand, *RfCht4* is the largest gene for chitinase from the RPW found in this study. The gene is 9,058 bp long with an ORF of 8,538 bp that encodes a putative protein of 2,846 amino acids. The identified sequence of *RfCht4* lacks a signal peptide but it contains five GH18 catalytic domains in which five CIDs are embedded, and has five CBDs. One CBD located between the first and the second catalytic domains, three CBDs located between the second and the third catalytic domains, and one CBD located between the fourth and the fifth catalytic domains (Figure 1 and Table 2). In comparison, *RfCht5* is the smallest gene among the *RfChts* identified in this study. *RfCht5* consists of 1,595 bp with an ORF of 1,344 bp that encodes a putative protein of 448 amino acids (Table 2). It contains a predictable signal peptide, a single GH18 catalytic domain but lacks

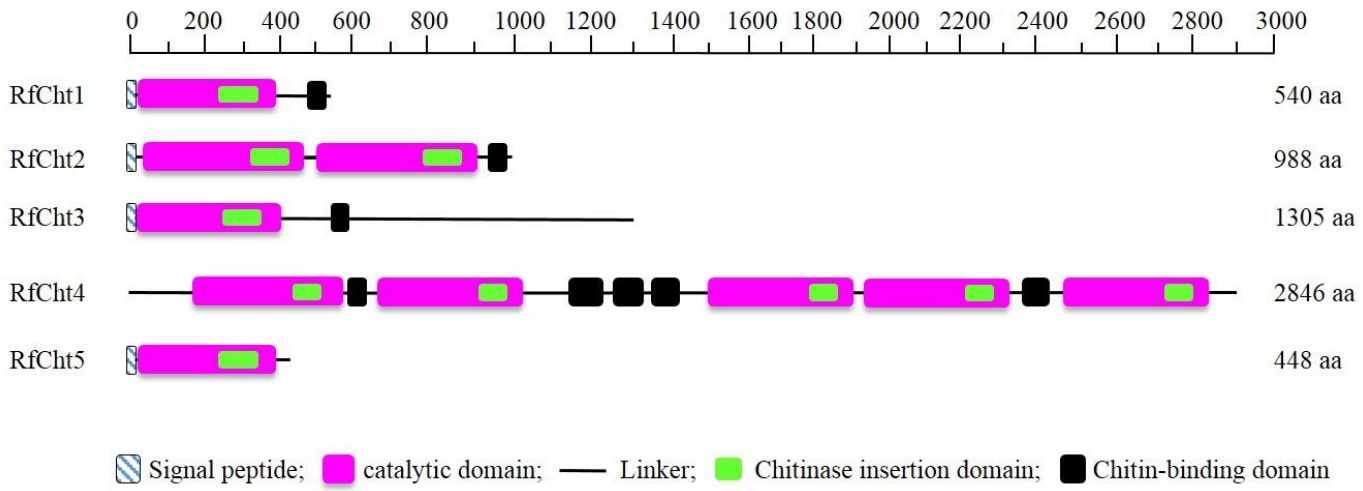

Figure 1. Domain organization of putative chitinases from RPW. The signal peptide was predicted by SignalP 4.1 server. The other domains were analyzed by the program InterPro. All investigated *RfChts* except *RfCht5* possess single or multiple chitin-binding domains (CBD) resembling the carbohydrate-binding module (CBM-14) found in peritrophins.

CBD (Figure 1). The five *RfChts* have many serine/ threonine (S/T) residues in the sequences between the catalytic domains and the CBDs. Similar to other insect chitinases, all CBDs of *RfChts* retain six conserved cysteines (C) that probably form three disulfide bridges that increase the affinity of chitinase for the insoluble substrate during chitin hydrolysis.

GenBank database homology searches and the sequence alignments revealed that the deduced amino acids of the five *RfChts* share common consensus signature sequences that identify them as GH18 chitinases (Figure 2) and these are well-characterized from many insects belonging to different orders and species (Zhu et al., 2008b; Su et al., 2016). All the five *RfChts* retain the signature sequences in the conserved region I (KxxxxxGGW) and in the conserved region IV (GxxxWxxDxDD) of GH18 chitinases. It is notable that the conserved region II (FDGxDLDWEYP) is common in all identified *RfChts* except *RfCht5* where the residue tyrosine (Y) was replaced by phenylalanine (F). Moreover, in the *RfCht4* the conserved glutamate residue (E) in the second catalytic domain was replaced by asparagine (N) (Figure 2).

Phylogenetic analysis of deduced protein sequences of five *RfCht* genes

A phylogenetic tree was constructed by using deduced amino acids of *RfChts* and sequences of chitinase and/or chitinase-like proteins from 21 insect species belonging to seven orders (Figure 3). The tree was generated using maximum likelihood method. Phylogenetically, *RfCht1* belongs to group I chitinases and is closely related to a hypothetical protein from the mountain pine beetle, *Dendroctonus ponderosae* (Keeling et al., 2013) and to chitinase 5 (*TcCht5*) of the red flour beetle, *T. castaneum* (Zhang et al., 2011a). *RfCht2*, *RfCht3*, *RfCht4,* and *RfCht5* phylogenetically belong to the groups III, VI, II, and VII, respectively (Figure 3).

Expression profiling of *RfChts* in the tissues and developmental stages

Expression profiles of *RfChts* primary transcripts in eggs, larvae, adults, and the appendages were determined by RT-PCR. Transcripts of *RfCht1* and *RfCht2* were expressed in the larval cuticles 96-h pre-molting and the expression continues to the onset of molting (Figure 4). *RfCht2* seems more steadily expressed 14-h pre-molting; however, 96- to 24-h pre-molting, fewer transcripts were expressed relative to the transcripts found 14- to 0-h pre-molting. The transcripts of both chitinases were found in the adipose tissues and the gut of larvae, as well as in the guts of both males and females, the elytra, and the hindwings (Figure 4). The transcripts of *RfCht3* were uniformly detectable 14- to 0-h pre-molting but in less quantity relative to *RfCht1* and *RfCht2* transcripts. *RfCht3* was not detected in cuticular tissues 96- to 24-h pre-molting and it was not found in adult weevils or their appendages. *RfCht4* transcripts were detected only in cuticles that were collected 96-, 8- and 4-h pre-molting. It seems there is no steady expression titer for *RfCht4* in the cuticle. Transcripts of *RfCht4* were not detected in adults or their appendages. Faint transcripts of *RfCht5* were detected in larval cuticular tissues 48- to 0-h pre-molting but were not found in other tissues and developmental stages.

Transcripts of the five chitinases were further

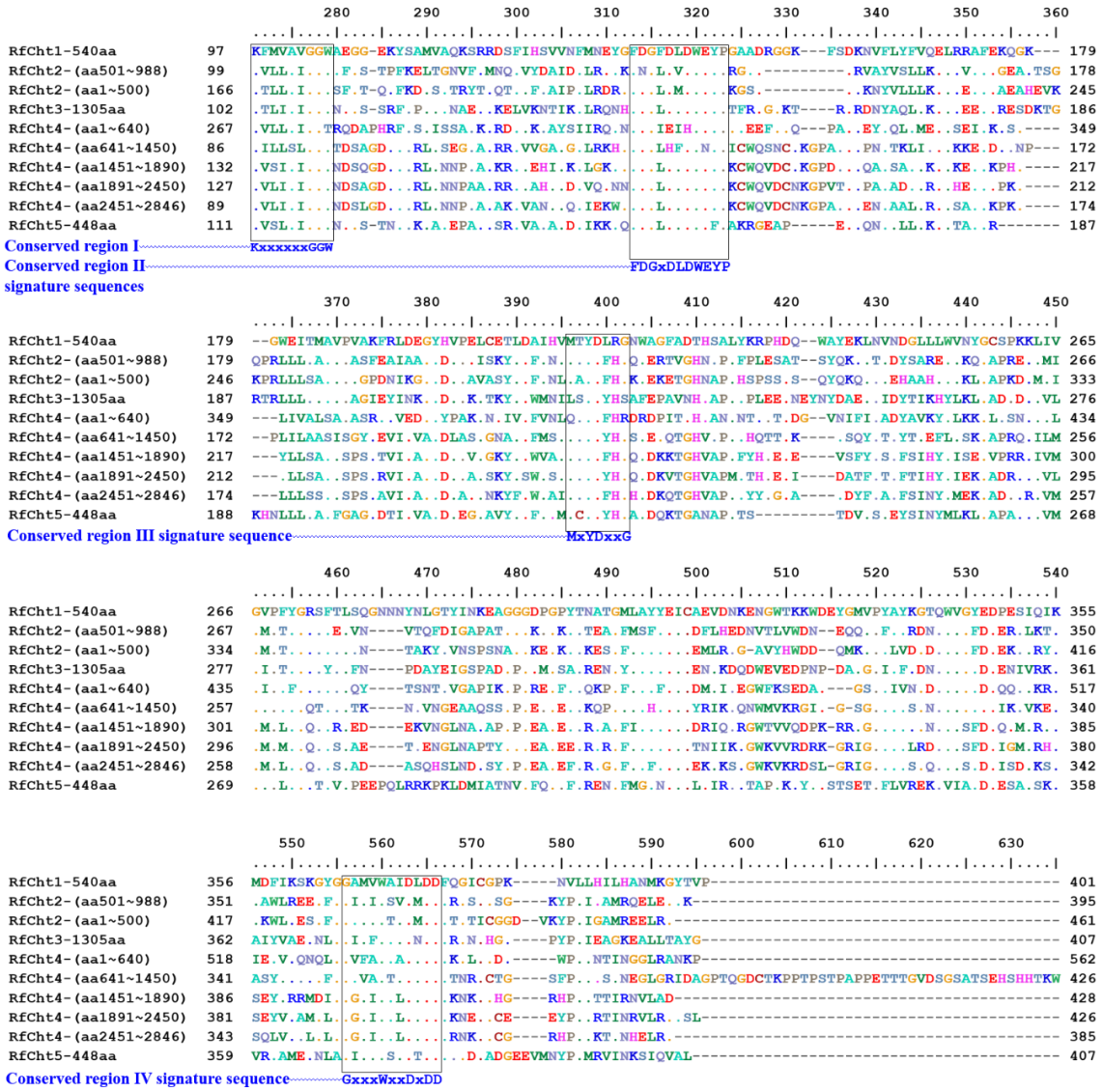

Figure 2. Multiple sequence alignment of the deduced amino acid for *RfChts*. MEGA7 (Kumar et al., 2016) and BioEdit softwares performed the multiple sequence alignment. Dots indicate identical amino acid sequences. Conserved regions I, II, III, and IV in insect chitinases were boxed. The signature sequences for each conserved region were also depicted.

investigated in the cuticles and internal organs of the larvae inhabiting the cocoon, pre-pupae, and pupae (Figure 5). *RfCht1* and *RfCht2* were found in all tested stages and tissues including adult wings, 12- and 24-h old eggs. *RfCht3*, *RCht4*, and *RfCht5* transcripts were very low relative to those for *RfCht1* and *RfCht2*. It is clear that the transcripts of *RfCht3*, *RCht4,* and *RfCht5* were steadily expressed but in low quantities in the last instar larvae within the cocoon, pre-pupae, and pupae. Transcripts of all the five *RfCht* genes were expressed in 24-h old eggs though more transcripts of *RfCht1* were expressed followed by *RfCht2*, *RfCht3*, *RfCht5*, and *RfCht4*. *RfCht2* transcripts were prominent in 12-h old eggs and hardly detectable for the other chitinases (Figure 5). Apparently, the transcripts of *RfCht2* were higher followed by the transcripts of *RfCht4*, *RfCht5*, *RfCht1*, and *RfCht3* in first instar larvae just emerged from the eggs (Figure 6).

DISCUSSION

Chitinases are indispensable enzymes involved in chitin metabolism leading to molting and eclosion of insects and other arthropods. The matrix polymer chitin has been designed to provide the cuticle with flexibility in response

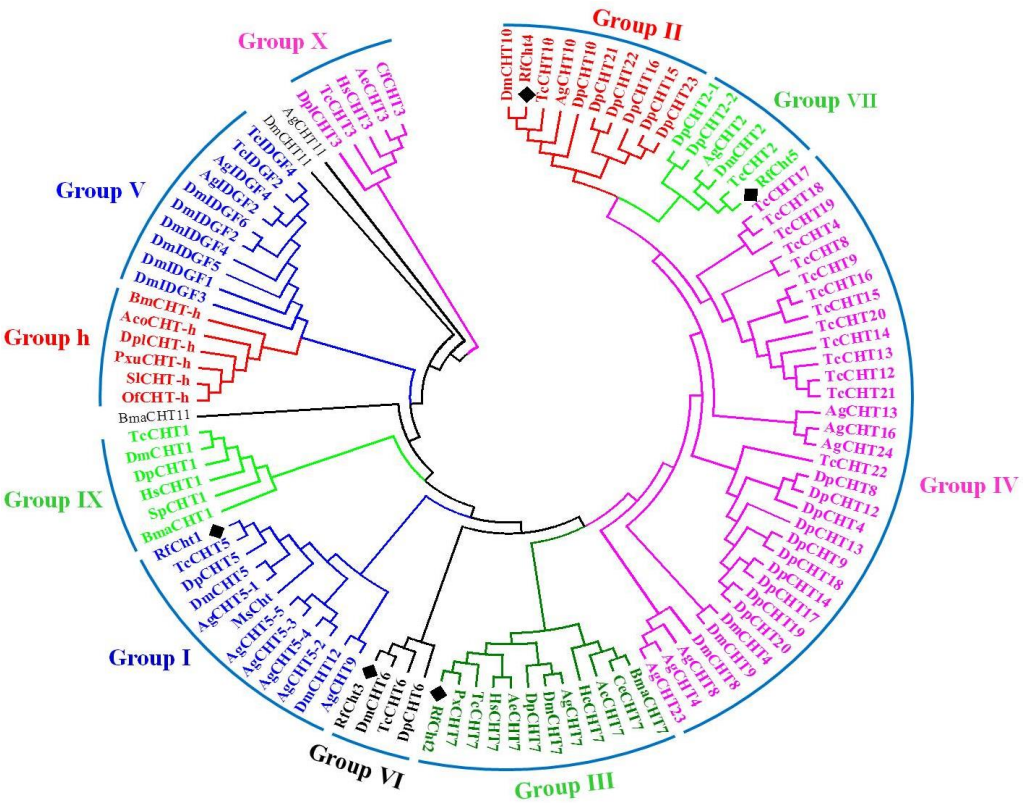

Figure 3. Molecular phylogenetic tree of *RfChts*. The tree was inferred by using the Maximum Likelihood method based on the JTT matrix-based model implemented in MEGA7. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 107 amino acid sequences downloaded from the GenBank. The sequences represent chitinases and chitinase-like proteins from 21 species of arthropods belonging to seven orders. *Acromyrmex echinatior* (Ae) – Hymenoptera; *Agrius convolvuli* (Aco) – Lepidoptera; *Ancylostoma ceylanicum* (Ac) – Nematoda; *Anopheles gambiae* (Ag) – Diptera; *Bombyx mori* (Bm) – Lepidoptera; *Brugia malayi* (Bma) – Nematoda; *Caenorhabditis elegans* (Ce) – Nematoda; *Camponotus floridanus* (Cf) – Hymenoptera; *Danaus plexippus* (Dpl) – Lepidoptera; *Daphnia pulex* (Dp) – Crustacea; *Drosophila melanogaster* (Dm) – Diptera; *Haemonchus contortus* (Hc) – Nematoda; *Harpegnathos saltator* (Hs) – Hymenoptera; *Manduca sexta* (Ms) – Lepidoptera; *Ostrinia furnacalis* (Of) – Lepidoptera; *Papilio xuthus* (Pxu) – Leidoptera; *Plutella xylostella* (Px) – Lepidoptera; *Rhynchophorus ferrugineus* (Rf) – Coleoptera; *Spodoptera litura* (Sl) – Lepidoptera; *Strongylocentrotus purpuratus* (Sp) – Echinodermata; *Tribolium castaneum* (Tc) – Coleoptera.

to the need to adapt to extensively diverse environmental stresses (Muthukrishnan et al., 2016). Insect chitinases have been characterized as potential targets for pest management either via directly targeting them (Su et al., 2016; Cao et al., 2017) or via manipulating their inhibitors as biopesticides (Arakane and Muthukrishnan, 2010; Hirose et al., 2010). *R. ferrugineus* lives and develops inside the palm trunk, where there are various biomass-degrading microbes that do not harm the weevil. In this context, it has been reported that polar surface cuticular extracts from adults and larvae inhibited the growth of Gram-positive bacteria and the entomopathogenic fungi *Beauveria bassiana* (Mazza et al., 2011).

Here, cDNAs have been isolated and synthesized for the chitinase genes from *R. ferrugineus* larvae that had not yet undergone apolysis. During this time the molting fluid fills the space between the digested old cuticle and the newly synthesized cuticle (Kramer et al., 1985). The identified chitinase genes shared the common consensus signature sequences found in all investigated insect chitinases, namely the conserved regions I, II, III, and IV that classify them into GH18. The largest gene for chitinase isolated from the RPW is *RfCht4* and the smallest one is *RfCht5*. The five *RfChts* identified in this study have divergent numbers of catalytic domains, CBDs, and CIDs that are believed to interact with oligosaccharides during catalysis (Li and Greene, 2010). The CID facilitates orienting and binding to longer chitin substrates when inserted into the triose phosphate isomerase (TIM) barrel, a conserved protein fold consisting of eight α-helices and eight parallel β-strands that alternate along the peptide backbone (Li and Greene,

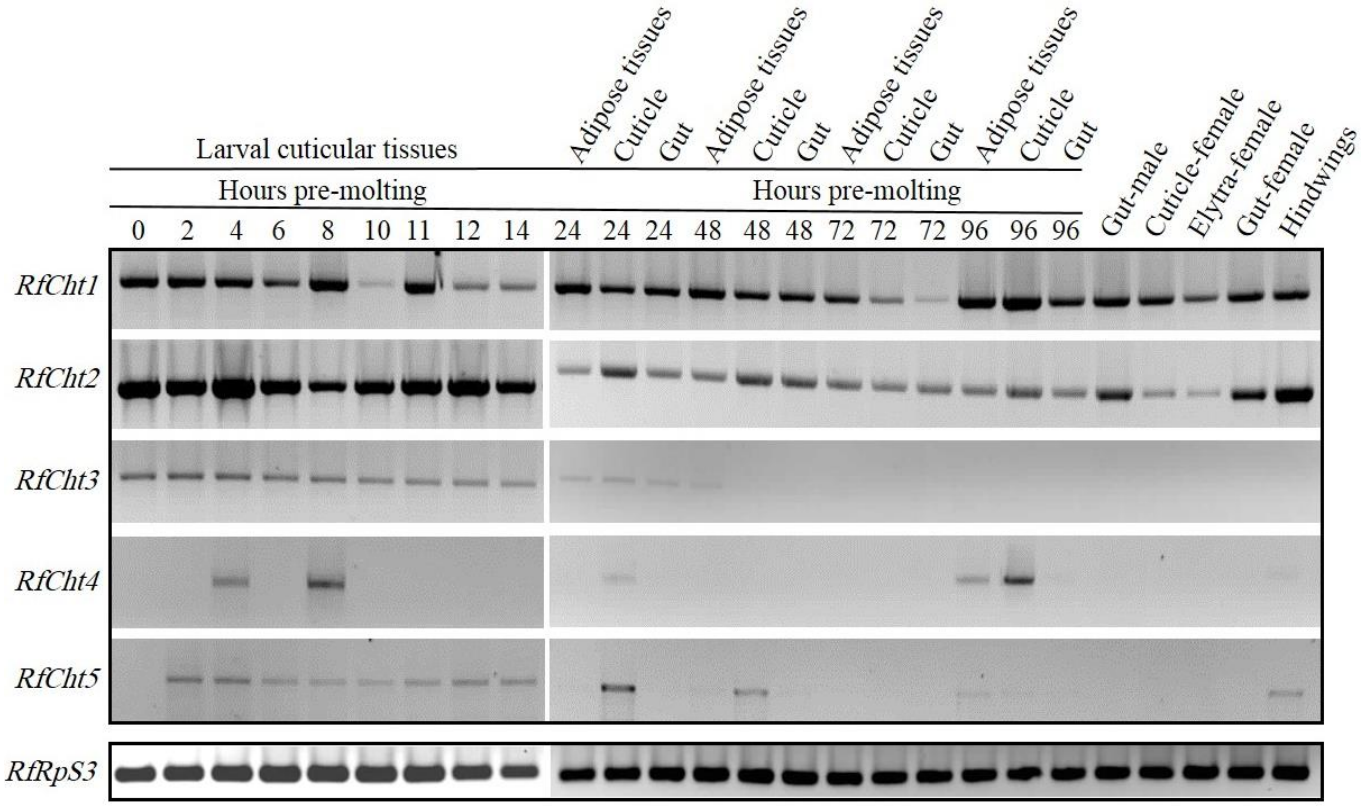

Figure 4. *RfChts* expression profiles in different tissues of middle-aged larvae and adults. The expression patterns of five *RfCht* genes were evaluated 96- to 0-hours pre-molting in larval adipose tissues, cuticles, and guts using RT-PCR. Likewise, the expression patterns were investigated in the adult's cuticle, gut, elytra, and hindwings. *RfRpS3* was used as reference gene for the RT-PCR.

2010). Moreover, with the exception of *RfCht5* that has no CBD, the CBDs of *RfChts* have linker sequences with six conserved cysteine residues. It has been reported that the common spacing between the conserved cysteines in the CBDs of GH18 chitinases appears to be as follows: $^{1}Cx_{11\text{-}24}$-$^{2}Cx_{5\text{-}6}$-$^{3}Cx_{9\text{-}19}$-$^{4}Cx_{10\text{-}17}$-$^{5}Cx_{4\text{-}14}$-^{6}C, where x is any other amino acid (Arakane and Muthukrishnan, 2010; Su et al., 2016). However, the six cysteine residues in *RfCht1* gene were arranged as follows: $^{1}Cx_{9}$-$^{2}Cx_{5}$-$^{3}Cx_{9}$-$^{4}Cx_{12}$-$^{5}Cx_{10}$-^{6}C. It is obvious that the spacing between the first and the second conserved cysteines in *RfCht1* CBD is only nine amino acids, that is, the spacing is shorter in *RfCht1* compared to the chitinase genes isolated from the RPW and from other insects. However, the spacing between the remaining conserved cysteines in the five *RfChts* investigated here is within the range commonly found in other insect chitinases. These linker regions can be heavily glycosylated and are believed to increase the stability of chitinases when present in a protease-rich environment such as the gut or the molting fluid (Abdel-Banat and Koga, 2002; Arakane et al., 2003; Arakane and Muthukrishnan, 2010).

Notable observations were found in the conserved region II (FDGxDLDWEYP) where the conserved tyrosine (Y) was replaced by phenylalanine (F) in *RfCht5* and the conserved glutamate residue (E) in the second catalytic domain of *RfCht4* was replaced by asparagine (N). These replacements were also observed in *CpCht10* from *Culex pipiens* and *PhcCht10* from *Pediculus humanus corporis* (Arakane and Muthukrishnan, 2010). Generally, glutamate residue (E) is the most critical in the conserved motif II and is believed to be the proton donor required for cleavage of the glycosidic bond as evidenced by the replacement of the residue (E) by a glutamine (Q) or with an aspartic acid (D) which resulted in complete loss of enzyme activity (Lu et al., 2002). Structurally, the identified *RfChts* typically resemble other known chitinases that identified from other insect species, especially in their physical and multi-domain architectures (Pan et al., 2012; Huang et al., 2012).

Insect chitinases and chitinase-like proteins are categorized into distinct groups according to phylogenetic kinships. Group I chitinases are secreted proteins that are the most abundant enzymes in molting fluid and/or integument, and represent the prototype enzyme of GH18, with a single copy each of the catalytic domain and chitin-binding domain (CBD) connected by linker polypeptide rich with S/T residues (Arakane and

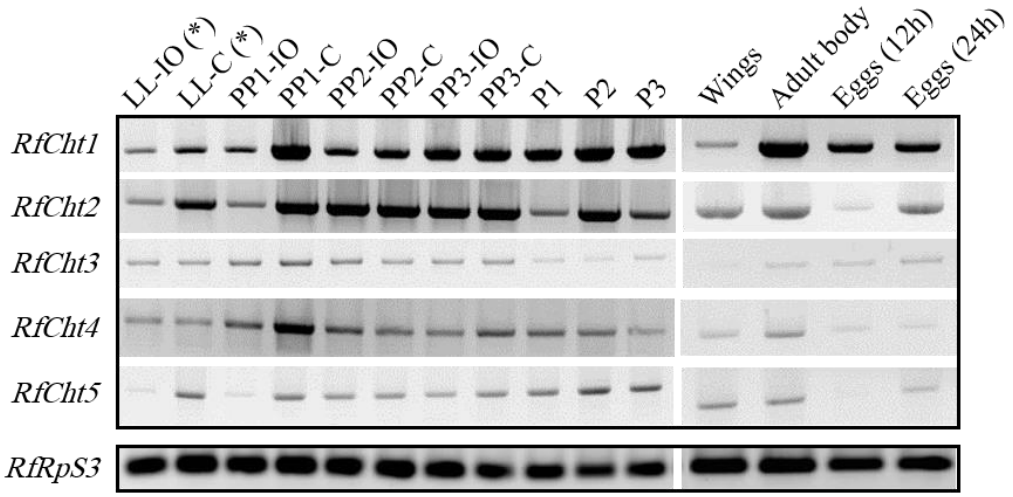

Figure 5. *RfChts* expression profiles in eggs, tissues of last instar larvae, pre-pupae, pupae, and adult weevil. The expression profiles were tested in cuticular (C) and internal organ (IO) tissues from the last instar larvae (LL) that have spun the cocoon (*) and inhabited inside, from three pre-pupae (PP), and from three pupae in addition to 12-hour and 24-hour old eggs. The expression was also evaluated in the adult's body as well as the wings. *RfRpS3* was used as reference gene for the RT-PCR.

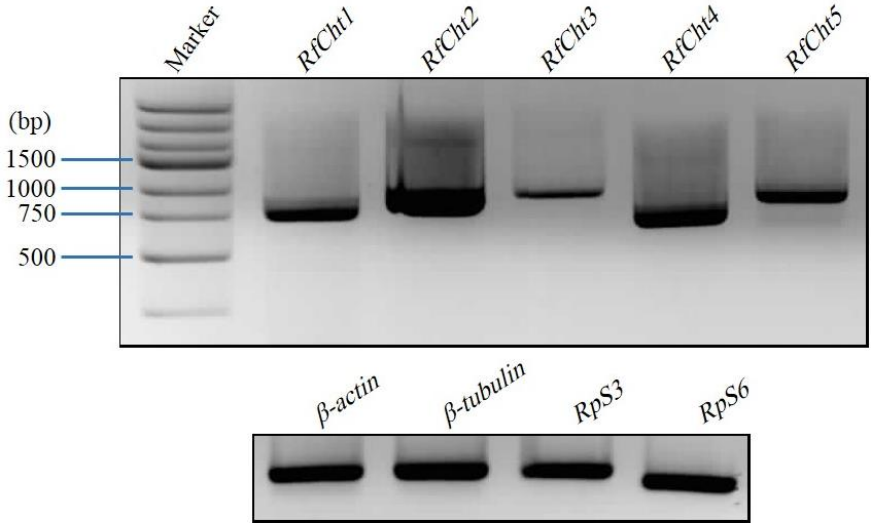

Figure 6. Evaluation of the primary transcripts for *RfChts* in the first instar larvae. The RT-PCR analysis was done to evaluate the expression of the five *RfChts* in tissues of the first instar larvae that instantly hatched from eggs. The housekeeping genes *Rfβ-actin*, *Rfβ-tubulin*, *RfRpS3*, and *RfRpS6* were used as reference genes for the RT-PCR.

Muthukrishnan, 2010; Khajuria et al., 2010; Tetreau et al., 2015). Previous studies reported that group II chitinases are larger-sized secreted proteins with multiple catalytic domains and CBDs (Zhu et al., 2008c; Tetreau et al., 2015). Group III chitinases contain two catalytic domains and are predicted as membrane-anchored proteins. Group IV chitinases are the most divergent. They usually lack a CBD and/or an S/T-rich linker domain and are predicted to be secreted proteins found in the gut or fat body. Group V proteins include the putative chitinase-like imaginal disc growth factors (IDGFs). In *T. castaneum* and *D. melanogaster* genomes, multiple genes were found to encode groups IV and group V chitinase-like proteins. In contrast, groups I, II, and III are each

represented by only a single gene in each species (Zhu et al., 2008c; Tetreau et al., 2015). Group VI chitinases resemble group I chitinases; however, the C-terminal S/T-rich linker extends the molecular mass of proteins in this group. Group VII chitinases structurally resemble group IV but phylogenetically are placed as an outlier of group II (Merzendorfer, 2013). The group has an N-terminal signal peptide and a GH18 catalytic domain, but it lacks a CBD.

The expression patterns of *RfChts* are similar to the expression patterns of a chitinase-like gene cluster (*AgCht5*) from the African malaria mosquito, *A. gambiae* (Diptera: Culicidae), where the five genes in the cluster showed different expression patterns at different developmental stages (Zhang et al., 2011a). It has been reported that expression of insect chitinases is time controlled and released only during molting. Chitinase transcripts are not detectable in cuticular tissue until after the cessation of feeding in each larval instar and appear immediately prior to pupation before dropping to undetectable levels 1 to 2 days into the molt cycle (Muthukrishnan et al., 2016). In contrast, our study showed that transcripts of *RfChts* were detectable in all stages at earlier times before the larvae had commenced the molting. Similar expression patterns were reported in *N. lugens* chitinase-like gene family (Xi et al., 2015). This suggests that at least some RPW chitinases may have been enrolled in functions other than molting. Due to the concealed living behavior of the weevil within the trunk of the host plant, a place where many species of microbes survive and propagate, it is probable that some of the chitinases might be involved in the immune defense to protect the weevil from microbial infection.

Molecular characterization and phylogenetic studies of five *R. ferrugineus* chitinases revealed RPW conserved the functional domain consensus commonly found in all identified chitinases from insects belonging to different orders. However, the expression pattern of some chitinases in the tissues of *R. ferrugineus* and developmental stages is unique. Expression of RPW chitinases varies from being in short time and in specific patterns usually found in other insects to an unusual constitutive expression throughout the larval developmental stages. This might be related to the living habitat of this insect pest, where an array of microbial inhabitants exists in the same habitation. Further studies are necessary to understand the specific roles of the constitutively expressed chitinases in *R. ferrugineus*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Bioactivity guided fractionation of potent antiacne plant extract against *Propionibacterium acnes*

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***Propionibacterium acnes* (*P. acnes*) is a commensal anaerobic, Gram positive bacterium that belongs to the normal microflora. *P. acnes* play an important role in the pathogenesis of various skin infections and diseases. However, the available agents are associated with number of side effects and *P. acnes* show the genetic base of antibacterial resistance against erythromycin and clindamycin. Therefore, alternative natural approaches for the treatment are needed for an hour for alleviation of infection caused by *P. acnes*. To find out the most effective anti *P. acnes* extract amongst the selected four plants commonly used for anti-acne potential and to obtain the characterized fraction of the extract with highest anti *P. acnes* potential, *in vitro* antimicrobial activity was evaluated using disk diffusion method and the zone of inhibition was compared using Hi-media zone measuring ruler. Afterwards, minimum inhibitory concentration (MIC) was determined for the potent fraction and the results were compared with Clindamycin. The active fraction was characterized chemically and its thin-layer chromatography (TLC) finger printing profile was prepared in order to standardize it. The methanolic extracts of *Rubia cordifolia* showed maximum (14.0 and 24.0 mm) anti *P. acnes* activity. The petro-ether fraction showed highest potential compared to other fractions of the extract. Sub-fraction D of petro-ether fraction with maximum activity was further purified. But, the results were similar to the sub-fraction D. The MIC was found to be 3 µg/ml of sub-fraction. *R. cordifolia* extracts have significant anti *P. acnes* activity in comparison to *Tephrosia purpurea*, *Viola tricolor* and *Serenoa repens*. The fractionation of *R. cordifolia* extract enhanced the activity but only up to certain extent. The TLC finger printing profile and chemical identification tests can be used for the identification/standardization purpose of this active fraction.**

Key words: *Rubia cordifolia*, *Tephrosia purpurea*, *Viola tricolor*, *Serenoa repens*, antibacterial activity.

INTRODUCTION

Propionibacterium acnes is an anaerobic, Gram positive bacterium that belongs to the normal microflora. *P. acnes* play an important role in the pathogenesis of various skin infections and diseases (Purchiaroni et al., 2013, Leheste et al., 2017). It lives primarily on, among other things, fatty acids in sebum secreted by sebaceous

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glands in the follicles (Beylot et al., 2014). This bacterium is largely commensal and just largely detectable on the skin of healthy preadolescents. Among different types of microbial populations, *P. acnes* is the predominant member in the skin areas of back, face and chest and may also be found throughout the gastrointestinal tract in humans (Aiyelaagbe et al., 2007) and many other animals. Its populations cover 50% of the human skin normal flora; however, the number of populations differs in different parts of the body. It ranges from less than 10 cells/cm^2 on the nose to 107 cells/cm^2 on the human facial skin. Besides, this bacterium may act as a skin pathogenic microorganism, which may lead to different skin diseases like acne vulgaris (Behazadi et al., 2016) which is a chronic inflammatory skin condition classified by the Global Burden of Disease Study as the eighth most prevalent disease worldwide. Acne develops as a result of increased sebum production; hyperkeratinisation, increase in *Propionibacterium* acnes, and inflammation (Omer et al., 2017). The inflammation and over-production of sebum caused by *P. acnes* can be overcome by topical and systemic antibiotic therapy. However, due to side effects like rashes, swelling, redness, irritation, dizziness and more importantly antibacterial resistance etc., this therapy needs to be replaced by some alternative solutions (Jończyk-Matysiak et al., 2017). Due to the increasing failure and rapid development of multi resistant bacterial strains of clinically important medical pathogens required, the development of newer antimicrobial agents comes with broadened horizons (Achermann et al., 2014). Therefore, plant based preparations have attracted attention for the treatment of bacterial diseases (Farnsworth, 1966; Julianti et al., 2017). Historically, plants have always remained a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two-fold in the development of new drugs; they may become the lead for the development of a medicine and a phytomedicine to be used for the treatment of diseases (Gilani et al., 2010). Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world. Many herbs comprise remarkable properties and functions on multiple biochemical pathways capable of controlling several organ systems simultaneously.

Nowadays, majority of scientist are intensifying their research on various herbs or combination of herbs. These plants are supposed to have extraordinary, unique, antimicrobial activity. For example different parts of various plants are being used for treatment of skin infections such as acne caused by *P. acnes.* Herbs are selected and combined for their ability to inhibit microbial growth in various part of the body and support organ systems responsible for detoxification and immune function. Phytoconstituents such as flavonoids, alkaloids, tannins and triterpenoids, obtained from medicinal plants challenges the modern medicine and stimulating opportunity for the expansion of modern chemotherapies against wide range of microorganisms (Gorle and Patill, 2010; Gupta et al., 2008; Altemimi et al., 2017). Less availability and unaffordable cost of new generation antibiotics initiated the search for alternative phytomedicine with claimed antimicrobial activity. The extractable bioactive compounds in medicinal plants are a significant alternative approach to synthetic antibiotics, which could be used as valuables in human disease management. Many herbs with significant antimicrobial activity have been reported in different traditional literatures (Hay and Adriaans 2004; Iwu, 1993; Farhat et al., 2013). The current work is aimed at showing the step by step evaluation of four antiacne plants on the basis of indigenous literature to find the effective drug source against *P. acnes* followed by bioactivity guided fractionation. The objective of this work is to find out the most effective anti *P. acnes* extract amongst the four selected plants (*Rubia cordifolia*, *Tephrosia purpurea*, *Viola tricolor*, *Serenoa repens*), and to obtain the characterized fraction of the effective extract with highest anti *P. acnes* potential. Manjistha (*R. cordifolia*) can be proven to be a best remedy to cure acne, due to its Varnya, Raktashodhak, Vishaghna, Rasayana, Krimighna properties. It is an Ayurvedic herb mentioned in *Charaka samhita* as *varnya* and *vishaghna* and in *Sushruta samhita* it is categorized as *pittasam samana*. Manjistha (*R. cordifolia*) can be proven to be a best remedy to cure acne, due to its Varnya, Raktashodhak, Vishaghna, Rasayana, Krimighna properties (Meena, 2015). Traditionally, the plant and its leaves are used to treat skin diseases, bacterial infections, snake bite, antioxidant, etc. Review of scientific literature suggests that plant extract has anti-cancer, anti-inflammatory and anti-acne potentials. The hydro-alcoholic extract of the leaves has been proven for anti *P. acnes* activity, with results more prominent in comparison to Clindamycin. In another study the methanolic extract significantly exhibited anti *P. acnes* activity (Gorle and Patill, 2010).

T. purpurea is the plant which is traditionally used to cure blood related disorders, bronchitis, boils, pimples and bleeding piles (Negi et al., 2015). In Ayurveda, various parts of the plant is used to treat impotency, tumour, pimple, asthma etc (Upadhyay et al., 2010). The leaves and its various extracts have been reported for anti bacterial (Gupta et al., 2008; Nasri et al., 2015), anti-inflammatory, analgesic and antioxidant potentials. The methanolic extract of the whole plant is reported to be effective against gram +ve and gram -ve microbes (Jayaweera, 1982).

Viola (*V. tricolor*) has traditionally been used as a topical home remedy for skin conditions like eczema and acne. In Ayurvedic terms viola is a blood-cleanser herb. The dried aerial parts of the viola are used in natural medicine preparations as it contains a number of

beneficial polyphenols, including salicylic acid - a known antimicrobial used in many homeopathic and commercial acne treatment products (ESCOP, 2009 Monographs: the Scientific Foundation for Herbal Medicinal Products). It is contemporarily used as a remedy for various ailments of skin since ancient times. In Bulgaria it is used for the treatment of cough, skin infections including acnes, dermatitis; and in Italy it is used to treat psoriasis (Witkowska et al., 2005; Nasri et al., 2015). The ethanolic extract of the whole plant displayed significant inhibitory activity against *Staphylococcus epidermidis, Staphylococcus aureus, Candida albicans, Bacillus cereus,* and *P. acnes* (Walter et al., 2011).

S. repens is traditionally used in several forms for the management of several skin diseases and bacterial infections. Crude *S. repens* extract was used by European and Americans for at least 200 years for the treatment of asthma, cough, tubercular laryngitis, weakness, bacterial infections, skin diseases, etc. When the Saw palmetto extract was combined with short acting antibiotic (Prulifloxacin), the extract showed increased efficacy of the therapy in chronic bacterial prostatitis tested in 210 patients. The extract has also been reported to possess anti-inflammatory activity (Ray et al., 2013).

The objective of this work is to find out the most effective anti *P. acnes* extract amongst the four selected plants, and to obtain the characterized fraction of the effective extract with highest anti *P. acnes* potential.

MATERIALS AND METHODS

Collection of samples

The plant material was freshly collected in the month of October from their natural habitats. *R. cordifolia* (leaves), *S. repens* (leaves) and *V. tricolor* (whole plant) were collected from Haldwani (Uttarakhand) and authenticated by Dr. S. K. Srivastava, Scientist D/HOO, Botanical Survey of India (B.S.I), Dehradun (Ref. No. BSI/NRC/TECH.(Ident.)/2013-2014/1032). The *T. purpurea* (leaves) were procured from Tirupati, Andhra Pradesh and authenticated by Dr. K. Madhava Chetty, Associate Professor, Department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh, India (Voucher specimen No. 1246). *P. acnes* Strain (MTCC 1951) was procured from (MTCC) IMTECH, Chandigarh.

Preparation of extracts and fractions

The plant materials were dried and ground to a coarse powder. The ground plant powder (300 g) was extracted with 80% methanol using a Soxhlet extractor for 48 h. The extract was filtered through Whatman filter paper, concentrated under reduced pressure, transferred to pre weighed China dish and stored in vacuum desiccators until constant weight was obtained. The antimicrobial screening of the extracts was laid down in order to obtain the purified mixture or potent compound with highest anti *P. acnes* activity. The extracts (*R. cordifolia* and *T. purpurea*) with highest anti *P. acnes* activity were successively partitioned with petroleum ether, ethyl acetate, chloroform, n-butanol and water separately. The fractions of these two potent antiacne extracts were subjected to antimicrobial testing using serial dilution method to ascertain the most effective fraction against *P. acnes*. The petro-ether fraction of *R. cordifolia* showed the highest activity. The fraction (100 mg) was then charged into column containing silica gel G as a stationary phase. The column was allowed to run with different mobile phases beginning from ethyl acetate to methanol for the purification of the fraction. This resulted in five different sub-fractions named A, B, C, D, E and F. These sub-fractions were further evaluated for anti *P. acnes* activity through disk diffusion method. Among them, sub-fraction D showed the most potent activity hence it was further fractionated by sub column which resulted into six sub-fractions (1, 2, 3, 4, 5 and 6) of D sub-fraction. These subfractions 1 to 6 were evaluated for their potential against *P. acnes* and the most potent sub-fraction 4 was characterized using chemical and TLC fingerprinting profile (Lutterodt et al., 1999).

Preparation of inoculums

Stock cultures were maintained at 4°C on slants of nutrient agar. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient agar medium and were incubated without agitation for 24 h at 37°C. The cultures were diluted with fresh nutrient agar broth to achieve optical densities corresponding to 2.0×10^6 (Cfu) colony forming units for bacteria.

Antimicrobial susceptibility test

All the extracts and fractions were screened against *P. acnes* (MTCC, 1951), obtained from IMTECH, Chandigarh (India). The disk diffusion method was used to test the antibacterial activity of the plant extracts. Sterilized nutrient agar medium (20 ml) were poured for each bacterium into each sterilized Petri dish. The plates were allowed to solidify for 5 min and inoculums suspension was swabbed uniformly. The entire agar surface of each plate was inoculated with this swab, first in horizontal direction then in vertical direction, which ensures the uniform distribution of organism over the agar surface (Jones, 1996). The filter paper disks (6 mm in diameter) loaded with 1 and 10 mg/ml of solution prepared by using dry extract were placed on the surface of bacteria seeded agar plates; the compound was allowed to diffuse for 5 min and then the plates were incubated at 37°C for 24 h. Apart from this, Hexa-G antibiotic discs constituting six various antibiotics were used as standards. At the end of incubation, inhibition zones formed around the disk were measured with Hi-media zone measuring ruler. These studies were performed in duplicates.

Minimum inhibitory concentration (MIC)

For measurement of MIC, fresh cultures were grown in 5 ml nutrient broth tubes. Tubes were impregnated with 3, 5, 10, 15, 20 and 25 µg/ml of fractions of *R. cordifolia*. Each of the tube was inoculated with 0.1 ml of freshly growing cultures. Uninoculated tubes were kept as negative control and tube inoculated with bacteria and antibiotic clindamycin was considered as positive control. Tubes were incubated at 37°C with growth of cultures observed at 24 h and after 48 h of incubation. Minimum concentration that shows the minimum growth was taken as MIC (Kaur et al., 2010).

Determination of MIC

The *R. cordifolia* fractions were thereafter evaluated to determine MIC value. The broth dilution method was adopted by using 0.5% DMSO for diluting the fractions and was further incubated for 48 h.

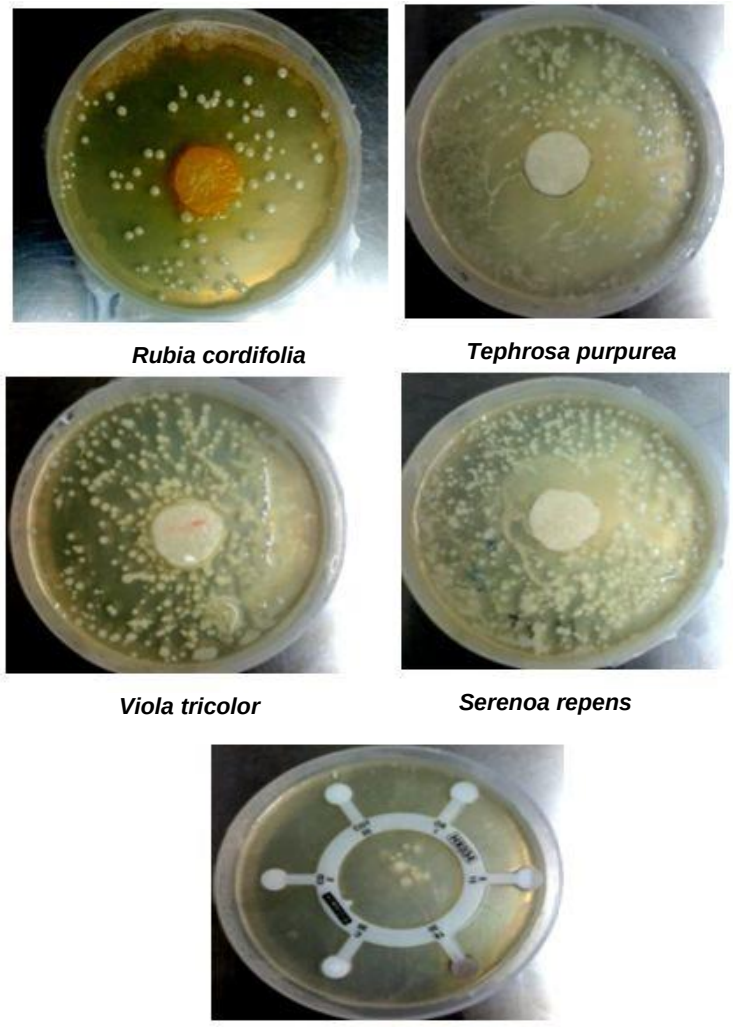

Figure 1. Antibacterial activity of various extracts in contrast to standards against *P. acnes* bacteria.

The minimum dilution of the plant fraction as regards killing of the microbes was observed.

Phytochemical analysis of fractions

The plant fractions were phytochemically screened using standard method. The fraction D and sub-fraction-4 were subjected to preliminary phytochemical screening for the detection of various phytoconstituents such as alkaloids, flavonoids, saponins, steroids and terpenoids (Koduru et al., 2006).

TLC finger printing profile

The thin-layer chromatography (TLC) finger printing profile of anti *P. acnes* fraction-4 was discovered for the identification/standardization purpose of active fraction. The mobile phase system comprised of toluene: ethyl acetate (8:2 v/v), was used.

RESULTS

Antibacterial activity

The antibacterial activity was determined by measuring the diameter of zone of inhibition recorded. The extract of the plant *R. cordifolia* was found to have maximum antibacterial activity in comparison to other three plants, that is, *T. purpurea*, *V. tricolor* and *S. repens*. The results obtained in the evaluation of the antibacterial activity of the different extracts against *P. acnes* are listed in Figure 1 and Table 1. The extracts had shown zone of inhibition

Table 1. Antibacterial activity of prepared all four extracts against *P. acnes*.

Plants	Concentration (mg/ml)	Zone of inhibition (mm)
Rubia cordifolia	1	14.0±1.5
	10	24.0±2.0
Tephrosia purpurea	1	12.0±2.0
	10	16.0±1.5
Serenoa repens	1	8.0±0.5
	10	14.0±2.2
Viola tricolor	1	6.5±0.5
	10	7.0±0.8
Clindamycin	1	16±2.0
	10	29±3.1

Among all the prepared extracts the maximum zone of inhibition was recorded in *Rubia cordifolia*, that is, 14.0 and 24.0 mm in concentration 1 and 10 mg/ml, respectively, followed by *Tephrosia purpurea* which has shown the zone of inhibition of 12.0 and 16.0 mm which is quite comparable to zone of inhibition of clindamycin, that is, 16.0 and 29.0 mm, respectively.

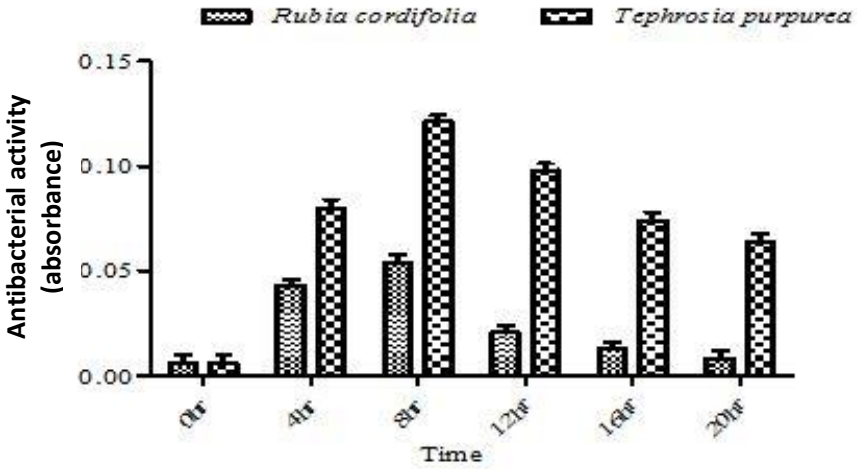

Figure 2. Antibacterial activity of petro-ether extract of *Rubia cordifolia* and *Tephrosia purpurea* (10 µg/ml).

against bacteria *P. acnes* but methanolic extract of *R. cordifolia* had shown maximum zone of inhibition against bacteria, that is, 14.0 and 24.0 mm in concentrations 1 and 10 mg/ml, respectively. Petroleum ether fraction showing the most potent antimicrobial activity in different dilutions with time interval of 4 h is listed in Figures 2 and 3. The sub fraction D that had shown highest anti *P. acnes* activity with zone of inhibition 26.0 and 32.0 mm at concentrations 1 and 10 mg/ml, respectively, are listed in Table 2. The fraction D was further fractionated with the help of sub-column. Six different fractions were collected out and evaluated for antibacterial activity. The results thus obtained were similar to that of fraction D and is listed in Table 3. The results for the phytochemical screening and TLC fingerprinting profile are listed in Tables 4 and 5. The minimum inhibitory concentration of sub fraction -4 was 3 µg/ml is listed in Table 6.

Minimum inhibitory concentration

The MIC of subfraction 4 was observed lowest among all

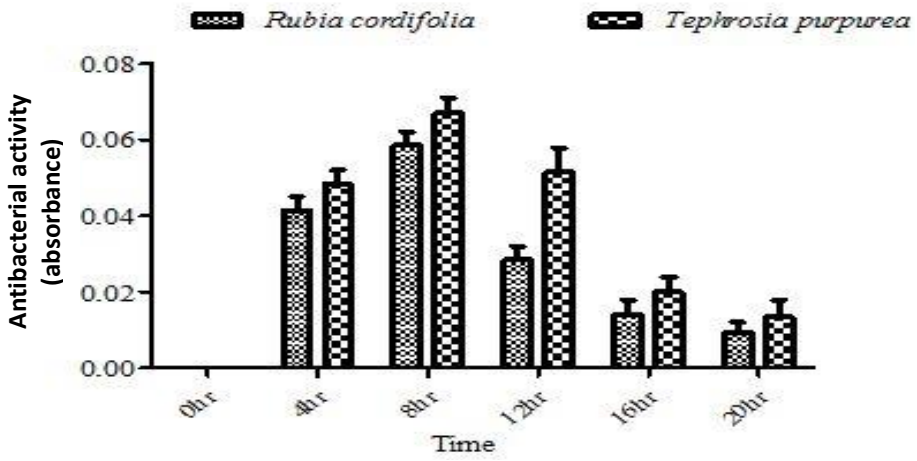

Figure 3. Antibacterial activity of petro-ether fraction of *Rubia cordifolia* and *Tephrosia purpurea* (25 µg/ml) against *P. acnes*.

Table 2. Antibacterial activity of different fractions of *R. cordifolia* extract against *P. acnes*.

Plants	Concentration (mg/ml)	Zone of inhibition (mm)
Fraction-A	1	16.0±0.8
	10	20.0±1.5
Fraction-B	1	12.0±0.4
	10	18.0±2.5
Fraction-C	1	19.0±1.9
	10	24.0±2.4
Fraction-D	1	26.0±2.7
	10	32.0 ±2.8
Fraction-E	1	11.0±0.5
	10	16.0±2.0

Among the five isolated fractions, fraction D showed the maximum zone of inhibition that is 26.0 and 32.0 mm in concentration 1 and 10 mg/ml respectively.

Table 3. Antibacterial activity of different sub-fractions isolated from fraction-D of *R. corifolia* extract against *P. acnes*.

Plants	Concentration (mg/ml)	Zone of inhibition (mm)
Sub-fraction 1	1	8.0±0.4
	10	11.0±0.3
Sub-fraction 2	1	12.0±0.5
	10	16.0±0.5
Sub-fraction 3	1	20.0±1.0
	10	24.0±1.2
Sub-fraction 4	1	25.0±0.7
	10	32.0±1.0
Sub-fraction 5	1	18.0±1.0
	10	22.0±1.1
Sub-fraction 6	1	16.0±0.2
	10	20.0±0.8

Among the six isolated sub-fractions of fraction D, sub-fraction 4 showed the maximum zone of inhibition that is 25.0 and 32.0 mm in concentration 1 and 10 mg/ml respectively.

the selected sub fractions, it was found to be 3 µg/ml.

DISCUSSION

The results obtained in this study indicate a considerable difference in antimicrobial activity between various extracts obtained which was determined by recording diameter of zone of inhibition. The activity of the plant *R. cordifolia* was more pronounced against the *P. acne* in comparison to other three plants, that is, *T. purpurea*, *Viola tricolor* and *S. repens* as listed in Figure 1 and Table 1. Till this stage, the anti-bacterial compounds of the plants assayed are not well known; however, the presence of flavonoids and terpenes and a certain degree of antibacterial compounds might be observed. Later, various extracts of *R. cordifolia* specifically petro-ether, chloroform, ethyl acetate and water were analysed for antibacterial activity and it has been observed that all the extracts had shown zone of inhibition against bacteria *P. acnes* but petro ether fraction showed the most potent

Table 4. Results of phytochemical screening of *Rubia cordifolia*.

Chemical	Tests	Results
Anthraquinone	Borntager's test	+ ve
Flavone	Shinoda test	+ ve
Saponins	Froth test	-ve
Alkaloids	Dragendorff's test Hager's test	-ve
Triterpenoids	Libermann Bruchard test	-ve

Table 5. R_f values under long UV (366 nm) and short UV (254 nm).

Plant name	R_f in long UV (366 nm)	R_f in short UV (254 nm)
Sub-fraction (4)	0.20, 0.25, 0.31, 0.38, 0.44, 0.51, 0.61, 0.69, 0.73, 0.81	0.21, 0.28, 0.35, 0.40, 0.46, 0.51, 0.65, 0.82, 0.89

Table 6. The result of zone of inhibition of sub fraction (4) at different concentrations.

Fraction	Concentration (µg/ml)	Zone of inhibition (mm)
Sub fraction-4	3	26.0
	5	Nil
	10	Nil
	15	Nil
	20	Nil
	25	Nil

antimicrobial activity in different dilutions with time interval of 4 h as listed in Figures 2 and 3. Furthermore, when petro-ether extract was further purified in a charged column, five sub fractions named as A, B, C, D and E were collected and their antimicrobial activity have been evaluated. The results showed that fraction D has maximum zone of inhibition, that is, 26.0 mm and 32.0 mm in the concentrations of 1 mg/ml and 10 mg/ml respectively. This anti *P. acnes* activity may attribute to the ability of the fraction to inhibit protein synthesis (Zhou et al., 2012), cell wall or nucleic acid synthesis (Gilani et al., 2010; Kaur et al., 2010). The fraction D was further purified with the help of sub-column. This purification resulted in the collection of six different fractions which were further evaluated for antibacterial activity. The results thus obtained were similar to that of fraction D listed in Table 3. When fraction-D was sub-fractionated, a decrease in antimicrobial activity has been observed which sucggests that later purification is not required. The higher activity of fraction D may be the result of complexes of components of the plant, relative to that of the sub fractions.

Our finding is in agreement with the finding of Witkowska et al. (2015) which reported the higher antibacterial potential of plant extract in the comparison with purified fraction. As the extract may comprise compounds of different polarity in comparison to purified fraction, that might be the reason for suggesting a synergism in antibacterial action between compounds of plants (Witkowska et al., 2005); otherwise, there will be portioning or purification. Moreover, due to the potent fraction (fraction-D) characterized by chemical and chromatographic methods, the anti *P. acnes* activity is supposed to be due to the presence of anthraquinone and flavones which are chief constituents of the plant (Lee et al., 2006). The above result opens the possibility of finding new clinically effective anti *P. acnes* drug and could be useful in understanding the relationship between traditional cures and current medicines.

Conclusion

The study concludes that methanolic extract of *R. cordifolia* leaves has significant anti *P. acnes* activity. Furthermore, the fractionation of extract enhanced the anti *P. acnes* potential. The most potent fraction was characterized by chemical and chromatographic

methods. The results of qualitative analysis of sub fraction confirmed the presence of anthraquinone and flavones which may be responsible for anti *P. acnes* activity of the sub-fraction. The above result opens the possibility of finding new clinically effective anti-acne drug and could be useful in understanding the relationship between traditional cures and current medicines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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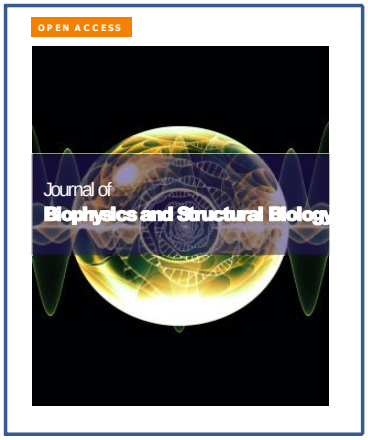

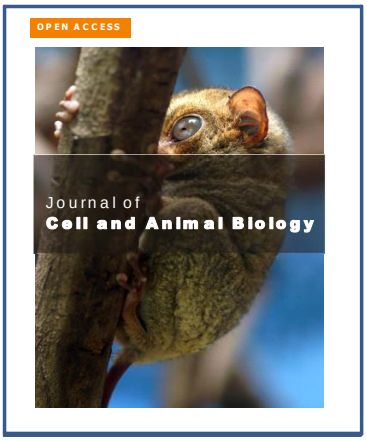

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