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Review

Ameliorative effects of betaine and ascorbic acid administration to broiler chickens during the hot-dry season in Zaria: A review

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This review highlights the huge challenges heat stress pose to profitable production of broiler chickens during the hot-dry season. It also enumerates the negative effects of heat stress due to excess production of reactive oxygen species (ROS) in broiler chickens. It emphasizes that administration of betaine and/or ascorbic acid is highly beneficial to broiler chickens, subjected to high ambient temperature and high relative humidity, characteristic of hot-dry season in the Northern Guinea Savannah zone of Nigeria. In conclusion, supplementation of betaine and ascorbic acid to broiler chickens during the hot-dry season may improve health and production, decrease mortality and increase their productivity.

Key words: Broiler chickens, lipid peroxidation, betaine, ascorbic acid.

INTRODUCTION

The hot-dry season in the Northern Guinea Savannah zone of Nigeria is thermally stressful to poultry (Sinkalu and Ayo, 2008). Meteorological factors, such as high ambient temperature and high relative humidity, exert adverse effects on poultry production (Chen et al., 2013). They are known to cause heat stress in poultry during the hot-dry season (Rhoads et al., 2013). It is considered that animals are under heat stress when there is an imbalance between heat production within the body and heat loss. Reactive oxygen species (ROS) are produced in excess of what the body can cope with under heat stress condition (Kikusato and Toyomizu, 2013). Exposure of broiler chickens to acute heat stress depresses the mitochondrial respiratory chain activity, which, in turn, leads to the excess production of ROS, and, consequently, induces lipid peroxidation (Yang et al., 2010). The increased ROS production induced by chronic heat stress occurs in the mitochondria of skeletal muscle cells through the elevation of membrane potential due to increase in oxygen consumption, especially at the initial phase of heat exposure (Azad et al., 2010b). This causes continuous lipoperoxidation of polyunsaturated fatty acids, and consequently, the destruction of cell membranes (Sunil-Kumar et al., 2011).

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The production of broiler chickens under heat stress conditions results in reduction of feed intake (Azad et al., 2010a). The decrease in live performance of birds reared under high ambient temperature, exceeding the thermo-neutral zone of the birds, is due to decrease in feed conversion to meat (Lagana et al., 2007). Heat stress damages the intestinal barrier in broilers due to oxidative stress (Gu et al., 2012), and negatively influences the welfare of broilers kept under pre-slaughter conditions (Vieira et al., 2011). It induces a rise in serum corticosterone concentration, mortality, and a reduction in the percentage of phagocyticizing macrophages. It causes mild multi-focal acute enteritis, demonstrated by an increase in concentration of lymphocytes and plasmocytes in the *lamina propria* of the jejunum (Quinteiro-Filho et al., 2012).

Antioxidant supplementation reduces the negative consequence of ROS activity such as ascites syndrome in broilers (Rajani et al., 2011). Betaine is an oxidative by-product of choline which serves as an osmo-regulator and is a substrate in the betaine-homocysteine methyltransferase reaction, which links choline and betaine to the folate-dependent one-carbon metabolism. Betaine is an important source of one-carbon units (Ueland, 2011). It has osmo-protective property and is a donor of methyl group (Hruby et al., 2005). Betaine is used by cells to defend against changes in osmolarity (Klasing et al., 2002).

Betaine supplement may stimulate protection of intestinal epithelium against osmotic disturbance; improve digestion, absorption and nutrient utilisation in broiler chickens (Mahmoudnia and Madani, 2012). Ascorbic acid is a potent antioxidant, as evidenced by the capacity to increase the level of enzymatic and non-enzymatic antioxidants of heat-stressed broiler chickens (Ismail et al., 2013). Ascorbic acid supplementation improves immunity of broiler chickens under heat stress conditions (Motasem, 2012).

The aim of the present review was to evaluate the ameliorative effects of betaine and ascorbic acid administration to broiler chickens during the hot-dry season in Zaria, located in the Northern Guinea Savannah zone of Nigeria.

POULTRY INDUSTRY IN NIGERIA

Agriculture, including poultry production is the most important sector of the Nigeria economy, providing employment for about 75% of the populace (CBN, 2007). In Nigeria, poultry production is a major contributor to animal protein. Nigeria has poultry population of 114.3 million, comprising 82.4 million chickens and 31.9 million other poultry, including pigeons, ducks, guinea fowls and turkeys (Abdullahi et al., 2006). The predominant systems of poultry production in Nigeria are subsistent and commercial systems. The commercial system is further sub-divided into small - (100 to 20,000 birds), medium - (20,001 to 50,000 birds) and large - (> 50,000 birds) scale poultry farming. These birds are kept under intensive system of management at a stocking density of 22 to 25 birds/m^2 (Dafwang, 2002). However, subsistent farming comprises indigenous breeds that are allowed to roam about to scavenge for feeds (Adeyemo and Onikoyi, 2012). In the tropical and subtropical regions of the world, including Nigeria, production performance of broiler chickens is adversely affected by high ambient temperature (Cahaner and Leenstra, 1992). Heat stress results in decreased feed intake, feed efficiency, body weight and flock activities.

These factors consequently result in increased mortality, increased pulse, respiratory and panting rates (Ubosi, 2001; Yunusa, 2002). Adverse effects of heat stress may result in 100% mortality in poultry farms in Nigeria (Obeng, 1985). Heat stress in broiler chickens has been associated with increased generation of ROS (Lin et al., 2006), ameliorated by antioxidants (Surai, 2006). Antioxidants such as ascorbic acid, singly or in combination with vitamin E, has been used to ameliorate the adverse effects of heat and transport stresses in Northern Guinea Savannah zone of Nigeria, during the hot-dry season in laying chickens (Ajakaiye et al., 2010) and broiler chickens (Onu, 2009).

POULTRY PRODUCTION AND ITS CHALLENGES

Production of chickens (*Gallus gallus*) for human consumption dates back to 4000 years ago. Since then, there has been a continuous selection for specific desired traits through selective breeding of parent stock to achieve the desired results (Kalmar et al., 2013). *G. gallus* is the major ancestor species, but *Gallus sonneratii* has also contributed to the genetic make-up of the modern domestic chicken. Furthermore, the knowledge of gene sequencing has accelerated the identification of causal mutations, determining major morphological differences between wild *Gallus* and domestic breeds (Job et al., 2011). The advantages of poultry production are ease of management, high turn-over, fast returns on investment, and its wide acceptance for human consumption (Haruna and Hamidu, 2004). Domestic chickens are also considered an important biological model for research in the biomedical field (Rubin et al., 2010).

The poultry enterprise is becoming complex as a result of the rapid strides in technology, changing market dynamics and growing scale of production. Due to some factors, starting from the procurement of chicks to their final disposal, entrepreneurs are faced with numerous constraints (Swu et al., 2012). Factors such as acute heat stress at marketing age, especially in broiler chickens

raised in open houses with poor ventilation impairing heat exchange, result in economic losses (Hassan and Reddey, 2012). Some considerations should be given to the microclimate within the broiler houses as birds experience heat stress (Lallo et al., 2012). Heat stress is one of the most important environmental stresses confronting poultry production worldwide. Understanding and controlling environmental conditions are crucial to successful poultry production and welfare (Lara and Rostagno, 2013). Some of the challenges faced in poultry production, especially in developing countries, include poor government support and management practices, high mortality and cost of feed (Amos, 2006). Evidence has shown that the critical issues of low production and inefficiency in resource allocation and utilisation in poultry production have adversely affected farmers in Nigeria (Ezeh et al., 2012). Population, urbanization and rising incomes are expected to double the demand for livestock products, including broiler meat in developing countries (Mammo, 2012). There is a shift in emphasis to broiler chickens for poultry meat, from spent layers (laying birds which have reached the end of egg production) in developing countries (Oluyemi and Roberts, 2000). There have also been significant improvements in poultry meat production in Nigeria due to efforts made in the use of improved breeds for production and the intensification of management systems of poultry (Ikani and Annalte, 2000). Poultry meat is affordable because it is relatively cheap for purchase by consumers (Damisar and Hassan, 2009). Broiler production is profitable because it has a positive net return on investment (Heidari et al*.,* 2011), whereas broiler meat has gained wide acceptance because it is a healthier alternative to red meat (Shini et al., 2010).

RELATIONSHIP BETWEEN ENVIRONMENTAL FACTORS AND THE WELFARE OF BROILER CHICKENS

The town of Zaria (11°, 12$^{/}$; 7°E, 38$^{/}$E) is located in the Northern Guinea Savannah zone of Nigeria. It has annual ambient temperature, ranging between 18.0 ± 3.7 and 31.8 ± 3.2°C, and has harmattan (the cold-dry period of the year) (November-February), hot-dry (March-May) and rainy (June-October) seasons (Ayo et al., 2011; Dzenda et al., 2013). The meteorological factors, such as ambient temperature and high relative humidity (particularly during the hot-dry season), directly influence production of broiler chickens (Genc and Portier, 2005). During hot conditions, characterized by high ambient temperature, relatively humidity and radiant energy, there is a decrease in the ability of animals to dissipate heat. This initiates compensatory and adaptive mechanisms to return the body to homeostasis (Daramola et al., 2012). When hot conditions persist, the difference between ambient temperature and body temperature of broiler chickens decreases, causing a reduced rate of sensible heat loss, which further results in mortality due to hyperthermia (Azoulay et al., 2011).

It is necessary to assess the environmental parameters of rearing broilers because they affect their performance (Kumar et al., 2011). For instance, heat stress, resulting from high ambient temperature and relatively humidity negatively affects poultry performance in the tropical and subtropical regions of the world (Sohail et al., 2010). The temperature-humidity index, an index of thermal comfort integrating the effects of ambient temperature and relative humidity (Purswell et al., 2012), is used to evaluate the degree of thermal stress in livestock (Dikmen and Hamsen, 2008).

THERMO-NEUTRAL ZONES OF BROILER CHICKENS

The thermo-neutral zone is the range of ambient temperature, which does not affect regulatory changes in metabolic heat production or evaporative heat loss in birds (Kingma et al., 2012). In the tropics, the diurnal ambient temperature fluctuations usually exceed the thermo-neutral zone of chickens, resulting in heat stress (Dei and Bumbie, 2011). Ambient temperatures outside the thermo-neutral zone of birds, irrespective of age, may negatively affect their energy balance and fitness (Ardia, 2013).

Metabolic disorders such as ascites and sudden death syndromes may occur in broilers reared above the temperature of thermal comfort in the first week of life of the birds (Fernandes et al., 2013). Broiler chickens subjected to heat stress show elevated corticosterone levels and lower levels of thyroid hormones (Mahmoud et al., 2014). Elevated temperature negatively affects production, reproductive potential, immune responses and health status of livestock (including broilers) (Nardone et al*.,* 2010). Holik (2009) reported that the thermo-neutral zone for poultry in the tropics is between 18 to 24°C and between 12 to 26°C in temperate regions, while Kingori (2011) showed that the most favourable temperature range for poultry is between 12 to 26°C. Broiler chickens exhibit different behavior in response to daily variations in the thermal environment. Wing-spreading and beak-opening are some important adaptations to thermal environments (Fernandes et al., 2013). Since adaptation of animal (including birds) to their thermal environment requires regulation of body temperature, measurement of that adaptation through the energy the animal expends, provides an indicator of the extent and energy cost of adaptation (Nienaber et al., 2009).

RESPONSES OF BROILER CHICKENS TO HEAT STRESS

The state of well-being of animals may be assessed by

the health, physiological and behavioral responses (Earley et al*.,* 2010). An increase in oxidative damage and alterations in amino acid concentrations in the diencephalon may contribute to the physiological, behavioral and thermoregulatory responses of heat-exposed chicks (Chowdhury et al., 2014). Behavioral responses such as posture, orientation, shelter seeking, huddling and dispersion are some means by which broilers seek to regulate their body temperature under hot ambient conditions (Kadzere et al., 2002). Heat tolerance in broilers may be enhanced by increasing sensible heat loss, which is determined by the difference between surface temperature and ambient temperature. It is more useful than the evaporative heat loss, which causes dehydration (Yahav et al., 2005). In addition to the damages that occur at cellular level due to oxidative stress, a number of signaling pathways, consisting of certain proteins (for example: apoptosis signal-regulating kinase 1, c-Jun NH_2-terminal protein kinase, signal transducers and activators of transcription) are modulated by heat shock protein 90 to improve tolerance to stress (Pandmini and Rani, 2011). Biochemical responses may be used to evaluate welfare of broiler chickens under hot conditions (Wang et al., 2013b). Heat tolerance in chickens may be assessed using biochemical parameters, especially during the early period of heat stress, owing to the close association between heat tolerance and biochemical indices (Run-Shen et al., 2011). Elevated ambient temperature is a limiting factor to poultry production in hot regions (Melesse et al., 2011). Though broiler chickens, to some extent, may acclimatize to some levels of oxidative stress, resulting from heat stress (Pamok et al., 2009), they experience some organ damage due to lipid peroxidation caused by hyperthermia (Mete et al., 2012).

Physiological responses to heat stress, which may be determined by their genetic make-up (Felvet-Gant et al., 2012), include decrease in antioxidant capacity, increased respiratory rate and rectal temperature (Ali, 2010). Heat stress decreases feed consumption (Chowdhury et al., 2012) and, consequently, reduces weight gain and growth. This further constitutes a major challenge in broiler production in the tropics (Widjastati and Hernawan, 2012). Heat stress also causes high water and electrolyte excretion, which impairs the ability for heat dissipation and alters acid-base homeostasis (Sayed and Downing, 2011).

Certain hormones like glucocorticoids and catecholamines are released in response to stressful conditions to enable the body cope with stress (Mostl and Palme, 2002). Two regulators of neuronal hormone regulators, arginine-vasotocin and corticotrophin-releasing adreno-corticotrophic hormones (ACTH), in addition to plasma corticosterone are also involved in stress responses (Cornell et al., 2013). High-affinity mineralocorticoid receptors ensure the maintenance of homeostasis, while the low-affinity glucocorticoid receptors mediate the recovery from stress. Hence, it is important to maintain balance between these systems for cellular homeostasis, performance and health (Dekloet, 2004). Glucocorticoids cross the blood-brain barrier to interact with certain receptors, located mainly at the hippocampus and the frontal lobes, consequently influencing learning and memory (Lupien et al., 2007). Acute heat stress activates corticotrophic-releasing hormone, resulting in impairment of memory consideration (Roozendaal et al., 2002). Increased plasma corticosterone may also down-regulate testosterone and progesterone concentration in plasma of chickens, impairing reproduction in broiler breeders (Rettenbacher et al., 2013). Finally, heat stress may result in redistribution of body resources (such as protein and energy) at the expense of growth, reproduction, production and health, to ensure survival (Gupta et al., 2013).

BIOMARKERS OF HEAT STRESS IN BROILER CHICKENS

Modern commercial broilers appear to have a compromised immune status, higher mortality and lower resistance to stresses, like heat stress (Khan et al., 2012). Heat stress induces hyperthermia in poultry (Syafwan et al., 2011). The health status of animals may be evaluated by measuring haemotological parameters (Talebi et al., 2005). Skin temperature, measured by thermography could be used as an index of welfare for domestic birds (Marelli et al., 2012). Erythrocyte osmotic fragility (EOF) may be used as an indirect measure of lipid peroxidation (Minka and Ayo, 2013). Heterophil/lymphocyte ratio is used as an indicator of heat stress (Prieto and Campo, 2010). The EOF is an important biomarker of oxidative stress, which may be utilised as a biomarker for heat stress in laying hens (Sinkalu et al., 2014). It may be used in broiler chickens to diagnose oxidative stress, having been demonstrated to serve as a biomarker of transport-induced oxidative stress, under high ambient temperature conditions, and as a reliable method for diagnosis of oxidative stress in quails (Minka and Ayo, 2013) and rabbits (Ayo et al., 2014). Furthermore, high levels of cortisosterone and heat-shock protein 70 may indicate levels of heat stressed (Zulkifli et al., 2009), and could result in high intensity levels of fear responses, evidenced by long tonic immobility in heat-stress broiler chickens (Al-Aquil et al., 2009).

REACTIVE OXYGEN SPECIES AND THEIR ROLES IN HEAT STRESS IN BROILER CHICKENS

In the eukaryotic cells, mitochondria are the site of

aerobic energy production. Electron transfer from respiratory substrates is coupled to oxygen to produce ATP; however, this transfer may lead to the formation of oxygen radicals and other (ROS) (Venditti et al., 2013). ROS are also generated in the mitochondria of skeletal muscles of heat-stressed broiler chickens (Azad et al., 2010a; Kikusato et al., 2010). The continuous generation of ROS and the inability to manage their negative burden result in oxidative stress and cellular damage due to lipid peroxidation, occurring in the membranes (Singh et al., 2013). Disease ensues due to a decrease in immune function of the body (Ambrozova et al., 2011). The ROS-mediated damages of erythrocyte membrane results in haemolysis (Eroglu et al., 2013), which is responsible for erythrocyte fragility (George et al., 2012).

Damage to the cytoskeleton is common in neuronal cell death and is an early event in oxidant-induced cell injuries (Tiogo et al., 2011). Oxidative stress causes depletion in energy, accumulation of cytotoxic mediators and cell death (Lee et al., 2009). Excessive ROS production is involved in the pathogenesis of contractile dysfunction in heart-failure (Kubio et al., 2011) and in carcinogenesis (Quan et al., 2011), due to the fact that cancer cells (like in Marek's disease) require elevated ROS levels to maintain their high multiplication rate (Sosa et al., 2013). It is also implicated in intermittent hypoxia-induced hypertension (Del Rio et al., 2010).

REACTIVE OXYGEN SPECIES SIGNALING AND THEIR ROLE IN THE BODY

ROS, which can be generated by the activities of mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenase, uncoupled nitric oxide synthase and myeloperoxidase enzymes, play both physiological (such as cell growth and stress adaptation) and pathological roles (such as cellular damage and attenuation of cell function) at different levels (Sugarmura and Keaney, 2011). ROS are regarded as signaling molecules, propagating information regarding cellular pathways and the overall redox and cell metabolic activities in the mitochondria (Ghouoleh et al., 2011). ROS, under pro-oxidant conditions, are regarded as essential triggers and modulators of cell-signaling and cell behavior. Hence, antioxidant compounds may interfere with cell signal transduction by interrupting ROS at critical levels of signaling pathways (Lee et al., 2009; Leonarduzzi et al., 2010).

Thus ROS, hitherto considered a toxic by-product of aerobic respiration, are now known to be cardinal factors in cell-cell signaling because they play numerous signaling roles from bacteria to mammalian cells (Mittler et al., 2011). ROS signaling explains the mechano-transduction of calcium ion (Ca^{2+}) discharge in the heart, both in healthy and pathological states, depending on the rate of discharges. This offers the possibility for new therapies (Prosser et al., 2011). The interaction of the sphingosine - 1- phosphate and its receptors controls the assembling of progenitor cells through the stimulation of ROS signaling on bone marrow stromal cells, haematopoietic progenitors and stromal cell-derived factor 1 release (Golan et al., 2012).

ANTIOXIDANT SYSTEM OF THE BODY

In response to oxidative stress, organs and tissues possess distinct antioxidant systems. The knowledge of antioxidant defense systems serves as a guide for establishing the most effective nutrient supplementation to reduce oxidative stress. Such approach enhances bird's health and welfare, product quality, and increase economic returns of broiler production (Panda and Cherian, 2013). The body protects itself against the negative effects of ROS by two mechanisms, namely: through the regulation of membrane permeability and its antioxidant potentials (Lushchak, 2011). The copper-zinc superoxide dismutase enzyme is an important cellular defense against ROS (Klooppel et al., 2010). The tumor necrotic factor-α raises the basal levels of glutathione by up-regulating γ-glutamyl cystein synthetase synthesis and stabilising potentials in cells (Persson and Vainikka, 2010).

The suppression of nuclear factor erythroid 2-related factor 2 (Nrf 2), an important transcription factor in antioxidant regulation system, occurs during oxidative stress in poultry (Kim et al., 2012), as evidenced by changes in levels of activities of superoxide dismutase, catalase, glutathione and thiobarbituric acid-reactive substances (Liu et al., 2013). The skeletal muscle mitochondria of broilers produce superoxide anions during heat stress (Mujahid et al., 2005). The life-span of erythrocytes, which may function in antioxidant defenses, decreases by 50%, when the cells are exposed to excessive ROS production. This may be due to protein and/or amino acid degradation (such as tryptophan) in their cytoskeleton (Olszewska et al., 2012). It may further be due to post-translational modification of proteins, destroying the fate and functions of the erythrocytes (Pandey and Rizvi, 2013).

ANTIOXIDANT SUPPLEMENTATION IN BROILER CHICKEN PRODUCTION

The amelioration of hyperthermia in poultry, due to heat stress, could be achieved by reducing the thermal load via increasing the dissipation potentials, reducing heat production levels or altering the daily malproduction pattern (Syafwan et al., 2011). Supplementations with

anti-stress agents in poultry are used to alleviate negative effects of stressors, especially heat stress (Pandurang et al., 2011).

Antioxidants decrease the deterioration of meat quality due to lipid peroxidation and stabilize meat oxidation after slaughter (Yasin et al., 2012). They also improve erythrocytic indices of broiler chickens subjected to heat stress (Majekodunmi et al., 2013).

a) Ascorbic acid as an antioxidant

It has been demonstrated that supplementation with L-ascorbic acid, both singly and in combination with dl-tocopherol acetate, is beneficial to heat-stressed layer hens (Ajakaiye et al., 2011). Ascorbic acid may be supplemented at 40 mg/bird/day in drinking water to reduce significantly the impact of heat stress and improve the productivity of broiler chickens (Vathana et al., 2002), due to its ability to improve the breast meat of broilers under heat stress (Abioja et al., 2010). During heat stress, endogenous ascorbic acid, produced by the kidneys of birds is not sufficient to mitigate the negative effects of the stress.

The adverse effects, resulting from heat stress, include reduction in immunity, feed intake, weight gain, egg production, number of chicks per hen, hatchability of fertile eggs, and of egg and carcass quality. It may also cause mineral imbalance, increase in panting and mortality, hence, necessitating the administration of supplemental ascorbic acid (Abidin and Khatoon, 2013). It has been shown that the body temperature, which is the net effect of heat production and heat loss, is reduced in chickens administered with ascorbic acid during exposure to high environmental temperature (Mckee and Harrison, 2013).

The physiological and biochemical potentials of ascorbic acid, an electron donor, are due to its ability to donate one or two electrons, making it a potent reducing agent and an antioxidant (Du et al., 2012). Its supplementation alleviates the negative effects of oxidative stress (Sujatha et al., 2010). Ascorbic acid has been demonstrated to decrease lipid peroxidation, and it improves protein concentration and iron status of broilers (Wang et al., 2011).

Glutathione activity is important for the maintenance of ascorbic acid metabolism by regulating the expression of ascorbic acid transporter and function (Mardones et al., 2012). Its supplementation to broiler chickens enhances the metabolic response to heat stress (Imik et al., 2013); improving performance and decreasing antioxidant status due to heat stress (Sahin, 2003). 2-0-α-Glucopyranosyl-L-ascorbic acid, a derivative of ascorbic acid protects dermal fibroblast from oxidative stress and cellular senescence. Therefore, it may be used as an anti-ageing agent (Taniguchi et al., 2012).

b) Betaine administration in livestock production

Betaine, a trimethyl derivative of glycine, is present in animal tissues. As a methyl group donor (Dilger et al., 2007), it is involved in transmethylation reactions as it donates its labile methyl group during metabolism. Betaine, widely found in animals, plants (wheat bran, spinach), microorganisms and seafood (from marine invertebrates) is known to protect cells, protein and enzymes from environmental stress (like high ambient temperature, low water and high salinity), because it is an osmolyte (Craig, 2004). It is also a metabolite of choline degradation and exerts an osmoregulatory role in cells (Hruby et al., 2005; Ratriyanto et al., 2009); especially in intestinal cells during heat stress (Metzler-Zebeli et al., 2009). As an osmolyte, betaine regulates water balance, resulting in the stability of tissue metabolism, especially in the gastro-intestinal tract (Lipinski et al., 2012). Betaine decreases lipid peroxidation in the breast muscles of broiler chickens, and, hence, the quality of broiler meat (Alirezaei et al., 2012). It may also exert free-radical-scavenging ability against lipid peroxidation, as well as maintaining myocardial energy (ATP) status via sustaining the enzyme activities in the Krebs cycle (Ganesan et al., 2007).

Betaine supplementation reduces the abdominal fat and facilitates the even distribution of lipid in geese (Su et al., 2009). The effects of betaine on fat may be due to its influence on mRNA expression and the promoter CpG dinucleotide methylation profiles of chicken's lipoprotein lipase gene (Yi et al., 2009; Xing et al., 2011). It further influences levels of plasma homocysteine, which could be used as a marker of methyl deficiency (Lever and Slow, 2010). Plasma homocysteine is reduced with betaine administration (Atkinson et al., 2009). In the transfer of its methyl group, methioine, a key intermediate, is converted to S-adenosyl methionine, and subsequently, to homocysteine. Homocysteine and methionine accumulate during methyl-donor deficiency (like in betaine deficiency), making the metabolites potentially toxic to the body (Waldroup et al., 2006). It also induces expression of spot-14 (S_{14}), which responds to thyroid hormones, located in hepatic nuclei, and functions to relay hormone and nutrient-related signals to genes involved in lipid metabolism (Su et al., 2009).

Methionine is a limiting amino acid in poultry (Kalbande et al., 2009). It is involved in vital functions such as methylation reactions of DNA (Swennen et al., 2011) and histones (Tesseraud et al., 2011). Osmolytes, like betaine, decrease inflammatory responses due to hyperosmolarity. This is because a high osmolytic state triggers pro-inflammatory cytokine release and inflammation (Brocker et al., 2010). Betaine also prevents the up-reglation of HSP 70 (Oliva et al., 2011). Osmolyte accumulation is necessary for the viability of cells of renal medulla. This is because renal medulla is exposed to

diverse ionic and osmotic compositions in their environment, which may result in ROS production (Rosab-Rodriguez and Valenzaela-Soto, 2010). Betaine, also known as N,N- dimethylglycine, may be included at 1 to 10 g/kg of feed without inducing toxicity or impairing performance in broilers (Kalmar et al., 2012).

PHYSIOLOGICAL ROLES OF METHYLATION PROCESS IN THE WELL-BEING OF BROILER CHICKENS

The expression of HSP is influenced by high temperature, which may be strongly associated with the DNA methylation pattern in the HSP70 promoter (Gan et al., 2013). HSPs are methylated in response to stress. Methly donors, like methionine and betaine increase development of breast muscle as evidenced by the expression of myostatin, Myf_5, and Mef_{2B} genes (Wen et al., 2014). Traits for growth are vital in the poultry industry, and they have been shown to be related to DNA methylation of chicken muscle (Hu et al., 2012). DNA methylation down-regulates the expression of a growth factor receptor responsible for tumor formations (Luo et al., 2013). Furthermore, brain-derived neutrotropic factor, which determines the susceptibility or resistance of chickens to Marek's disease, plays some roles in neuronal survival, cholesterol metabolism, cell differentiation and tumor formation (Yu et al., 2009). Tumor formation in chickens could be mediated by epigenetic alterations and genetic variation via the modification of DNA methylation, catalysed by DNA methyltransferase (Yu et al., 2008; Luo et al., 2012).

Generally, DNA methylation is the key factor of gene suppression (Useni et al., 2009). It functions in gene silencing by methylation of specific gene promoter sequences of the host genome against retrovirus and transcriptional suppression of transgenes (Jang et al., 2013). The difference in the expression of gene-mediated epigenetic processes could result in broad phenotypic expression in animals. DNA methylation may influence the extent to which the gene expression varies and also the modification of epigenetic mechanism (Nätt et al., 2012).

Furthermore, methyl-donor deficiency results in liver steatosis and, consequently, in a metabolic syndrome due to hypomethylation of the organic cation transporter PGC-1α, reduced binding with peroxisome proliferators-activated receptor γ (PPAR-γ), co-activator-α and hepatic nuclear oxidation. This links methyl donor deficiency and epigenomic deregulation of energy metabolism (Pooya et al., 2012). Such deficiency may also result in increased risk of cardiovascular diseases due to hyper-homocysteinemia. Consequently, it increases prohibin and decreases α-crystallin β, which indicate mitochondrial injury and stress to the endoplasmic reticulum (Martinez et al., 2013). It is conceivable that the negative effects of heat stress on the sustainable growth of broiler production in the tropical and subtropical regions may be alleviated by betaine administration in drinking water (Mahmoudnia and Madani, 2012).

CONCLUSION REMARKS

Poultry production in the Northern Guinea Savannah zone of Nigeria is important in meeting the high demand for safe and wholesome poultry meat in Nigeria, and indeed Africa. Owing to high ambient temperature and high relative humidity, which induce heat stress, broiler chicken production is greatly hampered, especially during the hot-dry season. This is because heat stress increases production of oxidants, causing oxidative stress and lipid peroxidation of cell membranes. During heat stress, broilers respond physiologically and behaviorally to combat the negative effects of the stress. This channels useful energy of the birds to coping with heat stress by attempting to return the body to homeostasis at the expense of weight gain and, consequently, production. Heat stress causes decreases in feed intake, conversion of feed to meat, poor weight gain and high mortality, resulting in huge economic losses.

The administration of potent antioxidants, such as ascorbic acid and betaine, ameliorate the negative effects of heat stress. The properties of ascorbic acid as an electron donor, and betaine, as methyl donor and an osmolyte, make their administration to broiler chickens, raised during the hot-dry season, beneficial in heat stress alleviation.

It is concluded that supplementation of betaine (1 to 10 g/kg of feed) and ascorbic acid (40 mg/bird/day in drinking water) to broiler chickens during the hot-dry season may improve health and production, decrease mortality and increase their productivity. It is recommended that further studies be conducted to elucidate the molecular and genetic mechanisms underlying the responses of broiler chickens to oxidative stress due to heat stress, with the aim of combating its negative effects.

Conflict of Interests

The author(s) have not declared any conflict of interests

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Review

Plant natural products research in tuberculosis drug discovery and development: A situation report with focus on Nigerian biodiversity

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Tuberculosis (TB) remains a disease of global importance with approximately two million deaths annually worldwide. Effective treatment of TB has been hampered by the emergence of drug resistant strains of *Mycobacterium tuberculosis*. The global resurgence of TB and the development of multidrug-resistant tuberculosis (MDR TB) and extensively drug-resistant tuberculosis (XDR-TB), call for the development of new anti-tuberculosis drugs to combat this disease. Plant natural products have a proven global history of treating diseases and ailments. This review aims to provide a situation report of on-going global efforts to discover and develop anti-TB drugs from plants, including plants found within Nigeria's rich flora. For two decades, studies on different families and genera of the plant kingdom have shown the great potential of plants as antimycobacterial agents. These motifs, including those from within Nigeria's flora, are discussed. Chemists, biochemists and molecular biologists have also employed technological developments in separation methods, hyphenated techniques, high throughput assays and microarray analysis, to drive the drug discovery process. Nigeria, and indeed, Africa, needs to look inwards to solve the burden of tuberculosis, by tapping on its rich biodiversity, which the continent is endowed with. There is need for the government to be committed and actively fund anti-tuberculosis research.

Key words: Plant natural product, antimycobacterial activity, drug discovery, drug development, Nigerian flora, biodiversity.

INTRODUCTION

Tuberculosis (TB) has continued to be a major health concern all over the world being the leading cause of death from any single infectious agent. It is estimated that one-third of the world's population is infected with TB (Dye et al., 1999). The World Health Organization (WHO) reported that in 2012, an estimated 8.6 million people

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developed TB and 1.3 million died from the disease (WHO, 2013). Nigeria remains one of twenty-two high TB burden countries around the world. As a measure to tackle this global public health problem, the WHO came up, in 1995, with a standardized control strategy called directly observed treatment short course (DOTS), also endorsed by the International Union against Tuberculosis and Lung Diseases (IUATL), to detect and cure TB (WHO, 1996). Despite the implementation of this strategy, the incidence, prevalence and mortality rates of TB in Africa have continued to be on the increase, and this trend was forecast to continue to 2015 (Dye et al., 2005). Co-infection of HIV with TB has challenged DOTS as a sole TB control strategy for Africa (Corbett et al., 2006; De Cock and Chaisson, 1999). In 2006, the WHO launched the new "Stop TB Strategy", a 10 year plan for the control of TB (WHO, 2006), but the core of the strategy remained DOTS in essence. In Nigeria, the DOTS programme has been implemented in all states and local government areas in the country and 3,000 DOTS centres have been operating across the country since 2006 (Erah and Ojieabu, 2009). Despite these efforts, the programme has not been fully implemented, and moreover, it is beset by incidence of bacterial resistance.

EMERGENCE AND IMPLICATIONS OF DRUG-RESISTANT *M. TUBERCULOSIS*

Inadequate, incomplete, or improperly supervised treatment regimen, wrong prescription, and co-infection with HIV are responsible for the emergence of resistant strains of *Mycobacterium tuberculosis* (MTB) (Corbett et al., 2006). A particularly dangerous form of drug resistant TB is multidrug-resistant TB (MDR-TB), which is defined as a specific form of drug-resistant TB due to a bacillus resistant to at least isoniazid and rifampicin (first line drugs), the two most powerful anti-TB drugs (Smith and Moss, 1994). There have also been reports on the emergence of extensively drug-resistant tuberculosis (XDR-TB), which means resistance to rifampicin and isoniazid and to any fluoroquinolone, as well as to one of three injectable second-line anti-TB drugs, such as capreomycin, kanamycin and amikacin (Centre for Disease Control, 2006). MDR-TB and XDR-TB take longer to treat with second-line drugs, which are mainly bacteriostatic, and have a lower efficacy than first line drugs. Moreover, these second-line drugs are expensive, toxic, difficult to combine with antiretroviral drugs, and are unavailable in most of Africa (Dean et al., 2002; Carroll et al., 2012). With the emergence of these drug-resistant and new strains of TB, adverse side effects of existing treatments as well as the long treatment regimen, and the influence of HIV, it has become imperative that an urgent need exists for the discovery and development of new anti-TB agents (Newton et al., 2000). It is noteworthy that for the first time in more than Forty years, a new diarylquinoline drug- Bedaquiline, was recently approved by the Food and Drug Administration (FDA), as a component of a combination therapy for the treatment of MDR-TB (FDA, 2012). The understanding of the complete genome of *M. tuberculosis* is quite critical in TB drug discovery as there is a need for target-based discovery of novel anti-TB agents that can act on a site different from the currently known (Pauli et al., 2005; Chhabria et al., 2009).

NATURAL PRODUCTS IN DRUG DISCOVERY AND DEVELOPMENT

Natural product chemistry and organic synthesis as tools in rational drug design

Natural products (NPs) have played and continue to play a significant role in the drug discovery process. For a long period of human existence, NPs were the only form of therapy available for use by sick people or to maintain health. Drugs of natural origin have been classified as: i) natural products, ii) products derived semi-synthetically from natural products, and iii) synthetic products based on natural product models (Cragg et al., 1997). Natural product chemistry and organic synthesis are powerful tools for optimising leads and for generating new diversity from natural scaffolds. The amalgamation of both is an important strategy in rational drug design. Statistics from studies carried out by different workers continue to emphasize the potentials and untapped reservoir of molecules with therapeutic interest. Eighty percent of the world's population depends mainly on NPs for their health care and sixty percent of the orthodox drugs currently in use have their origin from NPs (Cragg and Newman, 2005). Evidence of the importance of NPs is provided by the fact that close to half of the bestselling pharmaceuticals in 1991 were either NPs or their derivatives (O'Neill and Lewis, 1993). Newman and co-workers (2003) reported that 61% of the 877 new chemical entities (with low molecular weights) registered as drugs worldwide during the period of 1981-2002 were or have been inspired by NPs. A total of 29 new NPs and NP-derived drugs were introduced in the United States, Europe and Japan between 2000 and 2003 (Burtler, 2004). More recent studies revealed that between 2005 and April 2010, some 19 NP-based drugs were approved for marketing worldwide (Mishra and Tiwari, 2011).

Natural products as leads in novel and active chemotypes

There is an urgent need to identify novel, active chemotypes as leads for effective drug development, and, as was dramatically illustrated by the discovery of the "wonder" antibiotics of the 1940s and 1950s, nature is the prime source of such lead discoveries. It has however been estimated that only 5 to 15% of the approximately 250 000 species of higher plants have been systematically investigated for the presence of bioactive compounds (Balandrin et al., 1993). Norman Farnsworth stated in his closing remarks in his guest editorial on "An old source for new drugs" in the August 1995 issue of Pharmaceutical Technology that "the world of plants represents a virtually untapped reservoir of novel drugs awaiting imaginative and progressive organisations" (Farnsworth, 1995). The success of NPs in drug discovery can be attributed to their high chemical diversity, biochemical specificity, greater number of chiral centres and increased steric complexity than either synthetic drugs or combinatorial libraries, and the effects of evolutionary pressure to create biologically active molecules by interactions with different proteins and biological targets (Wolfender, 2009; Queiroz et al., 2009).

Natural products and combinatorial chemistry

Despite the successes recorded, there was a global decline of the NP discovery programme by pharmaceutical companies in the 1990s. This was replaced by combinatorial chemistry, which, with the introduction of High throughput screening (HTS), became the preferred choice in drug discovery and development (Lee and Breitenbucher, 2003). This was based on the premise that combinatorial chemistry would generate libraries consisting of millions of compounds, which would be screened by HTS and produce drug leads by sheer number of molecules. In addition, it would also take care of intellectual property (IP) issues generated with NPs. This technology did not prove successful as results from early combinatorial libraries were often disappointing and only few drugs have been discovered by the combination of HTS and combinatorial chemistry (Burtler, 2004; Kingston, 2011). Interest in NPs was therefore renewed, as they have a proven history of providing medicinal agents and more so, they occupy a complimentary region of chemical space as compared with a typical synthetic compound library (Ortholand and Ganesan, 2004). To achieve a well-balanced drug discovery programme, there is no doubt that NPs play a pivotal role and therefore, must be incorporated into the programme. Combinatorial chemistry has improved over the years and NPs are used as starting templates in the synthesis of combinatorial libraries (Lee and Schneiber, 2001).

MEDICINAL PLANT RESEARCH AND DRUG DISCOVERY

Herbal medicine as an ancient practice

The use of plants and plant preparations for the treatment of diseases has been in existence since. Some of the earliest records of usage of plants as drugs are found in Artharvaveda, which is the basis for Ayurvedic medicine in India, dating back to 2000 BCE; the clay tablets in Mesopotamia, dating to 1700 BCE; and the Eber Papyrus in Egypt, dating to 1550 BCE (Sneader, 2005). In developing countries particularly in Africa, the population continues to rely on traditional medicine (TM) for their primary healthcare. It is estimated that up to 80% of the populations in Africa, Asia and Latin America depend mainly on TM for their healthcare needs, involving mainly the use of plant extracts (WHO, 2003). These extracts are used as herbal drugs in form of powders, concoctions, ointment, decoctions and infusions. The limitations of these herbal drugs revolve around lack of documentation, lack of standardization and quality control, dosage, and the common tendency to describe diseases and ailments vaguely (Okogun, 2002). In addition, some of these medicinal plants may be of rare existence, and difficult or impossible to grow and propagate. Therefore, it becomes even more challenging to identify and isolate the active principle/-s which would be needed for onward synthesis or derivatisation. Due to these challenges experienced with TM, modern science must be applied to the practice to ascertain the efficacy of the plants used. A guided methodological approach to the discovery of plant based drugs, as previously described by Queiroz et al. (2009); Hostettmann et al. (1997), involves the following steps:

1. Taxonomic identification of the plant.
2. Collection and drying of the vegetable material.
3. Extraction of plant materials using different solvents.
4. Fractionation of the extracts.
5. Analysis of extracts and fractions by a combination of chromatographic methods.
6. Purity control of the isolated compounds.
7. Structure elucidation of the constituents by a combination of diverse spectroscopic techniques (UV/VIS, IR spectrophotometry, carbon and proton nuclear magnetic resonance, mass spectrometry, X-ray diffraction) and chemical techniques (hydrolysis, formation of derivatives, degradation reaction etc).

8. Application of *in vitro* and/or *in vivo* screening models.
9. Pharmacological and toxicological assays (pre-clinical assays).

These steps require an inter-disciplinary approach amongst biochemists, ethnobotanists, molecular biologists, organic chemists, pharmacognosists, pharmacologists, taxonomists, and other scientists interested and involved in medicinal plant research. For example, in the drug discovery process, the ethnobotanist and taxonomist collect and identify the plant, respectively, the chemist and biologist work hand in hand to obtain the active principle responsible for a therapeutic activity. It is only after this identification and possible modifications that the drug goes down the chain of pharmacology and series of clinical trials, to establish its efficacy and safety, before it is accepted as a drug. These processes take at least ten years. Plant derived secondary metabolites (phytochemicals) continue to be an important source of new drugs for the following reasons; a) they find direct medicinal application as drug entities, b) they are useful as leads or templates for synthesis or semi-synthesis of structural analogues or derivatives c) they provide inspiration to organic chemists for drug design and total synthesis of new drug entities, d) they serve as biochemical/pharmacological probes and e) they can be used as small-molecule drug precursors which can be converted into the compound of interest by chemical modification (Salim et al., 2008; Harvey, 2008). Some excellent examples of the potentials of plant natural products and their synthetic/ semi-synthetic analogues are presented in Table 1.

Strategies for plant selection and compound isolation in drug discovery

The choice of plant genera and species for phytochemical and biological studies can be very difficult especially because the number of plants which have not been studied from this point of view is quite enormous. Selection of plants for the purpose of drug discovery can be categorized into four main groups:

1. Ethnomedicine
2. Field observations
3. Chemotaxonomic relationships
4. Random selection

Ethnomedicine/ ethnobotanical survey

This approach provides a useful way to discovery of new leads in the drug developmental process by relying on information provided by the traditional medical practitioners (TMPs). Farnsworth et al. (1985) have reported that at least 119 compounds derived from 90 plant species can be considered as important drugs currently in use in one or more countries, with 77% of these being derived from plants used in traditional medicine. In a similar study, Fabricant and Farnsworth (2001) identified 122 compounds from 94 plant species, which are used globally as drugs, with 80% of these having an ethnomedical use identical or related to the current use of the active elements of the plant.

Field observations

Secondary metabolites are produced as toxic materials for providing defence against predators, as volatile attractants for pollinators, or as colouring agents to attract or warn other species (Dewick, 2009). Environments such as the tropical forests, where the plants have to survive from all year the around attack of a diversity of other plants, bacteria, fungi, viral strains and insects, must be taken into consideration in the search for pharmacologically active natural products.

Chemotaxonomy

The chemotaxonomical approach in selecting medicinal plants to be studied provides an insight into the chemical composition and eventual isomers and analogues of biologically active compounds in different plant species of the same genus or family. This approach can be fruitful, as some secondary metabolites are found to be specific to a genus or a family. For example, compounds such as iridoids, triterpene acids, chlorogenic acid derivatives and flavonoids have been reported from members of the Rubiaceae family, such as *Adina racemosa*, *Galium verum*, *Galium tortumense*, *Saprosmas cortechinii*, *Morinda citrifolia* and *Asperula arvensis* (Itoh et al., 2003; Demirezer et al., 2006; Guvenalp et al., 2005; Ling et al., 2002). Observation of a biological/ pharmacological activity in a constituent of a species of the genus or family can lead to investigations into other species, to find analogues with similar activity.

Random selection

This strategy is especially useful when using high throughput screening techniques where a lot of extracts can be screened at a go. Research institutes such as

Table 1. Plant derived drugs of pharmaceutical value.

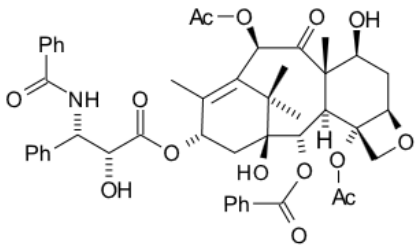
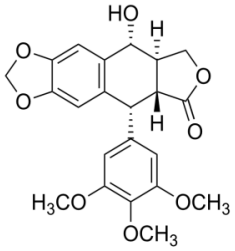
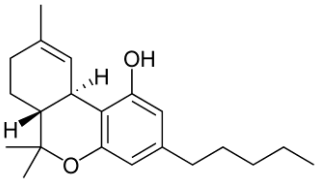
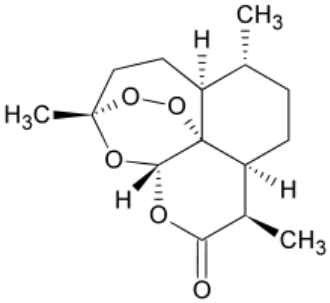
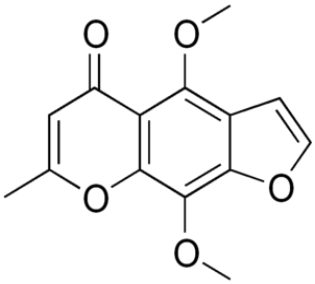
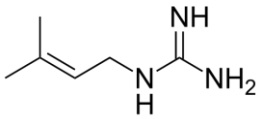
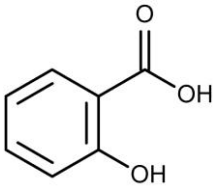
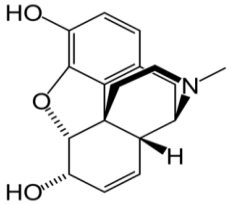
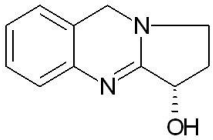
Botanical source	Isolated compound	Synthetic/semi-synthetic analogue	Therapeutic use
Taxus brevifolia L.	 paclitaxel	Docetaxel (Taxotere)	Anticancer
Podophyllum peltatum	 Podophyllotoxin	Etoposide	Anticancer
Cannabis sativa L.	 Δ^9-tetrahydrocannabinol	Nabilone	Nausea
Artemisia annua L.	 Artemisinin	Artesunate, Artemeter, Dihydroartemisinin	Antimalarial
Amni visnaga (L.) Lam	 Khellin	chromolyn sodium	Bronchodilator

Table 1. Contd

Galega officinalis L.	 Galegine	Metformin	Antidiabetic
Willows bark	 Salicylic acid	Aspirin	Analgesic, antipyretic, anti-inflammatory
Papaver somniferum	 Morphine	Methadone	Anaesthesia
Adhatoda vasica	 Vasicine	Bromhexine, ambroxol	Mucolytic agent/ bronchodilator

National Cancer Research Institute (NCI) at Frederick, Maryland, USA and Central Drug Research Institute, in India, have employed this approach. One of the successful stories from these studies is paclitaxel (Taxol), an anticancer taxane diterpenoid derived from the relatively scarce Pacific or Western yew tree, *Taxus brevifolia* Nutt (Kingston and Newman, 2007).

BIOLOGICAL EVALUATION OF ANTI-TUBERCULOSIS ACTIVITIES OF PLANT DERIVATIVES

General aspects of selecting bioassay methods

A major aim of investigations into plants and plant derivatives was to ascertain the biological or pharmacological effects, and this requires suitable bioassays for monitoring these effects. The term 'plant derivatives' is used for crude plant extracts, fractions and compounds, which may be used in modern phytotherapy (Ameh et al., 2010a, 2010b). In considering the various assay methods available, the guiding factors should be in systems that are simple, rapid, reproducible and inexpensive. For compounds with very low yields, the bioassay has to be sensitive enough for their detection. The number of false positives should also be reduced to a minimum. The complexity of the bioassay has to be designed as a function of the facilities, resources and personnel available. These factors are however determined by the choice of the target organism, depending on its virulence. Developments in automated high throughput screening programs have reduced the time lag experienced with screening of plant extracts. With advances in data handling systems and robotics, a hundred thousand samples can be screened within a week using the 384- well plate (Mishra et al., 2008).

Test organism

M. tuberculosis H_{37}Rv available from the American Type

Culture Collection (ATCC 27294) is the organism of choice for antimycobacterial investigations as it has a drug susceptibility profile fairly representative of most drug susceptible clinical isolates. The practicability of working with such a pathogenic organism though, makes this option difficult in many laboratories. This is because there are specific biosafety guidelines that demand the use of laminar-flow hoods and level 3 facility equipment for *M. tuberculosis* laboratorial work. Alternatives to this strain are other slow growing avirulent strains, *M. tuberculosis* H_{37}Ra and *M. bovis* BCG which are closely related to *M. tuberculosis* H_{37}Rv in terms of genetic composition and drug susceptibility profiles. Many researchers prefer to work with the rapidly growing, avirulent, saprophytic, surrogate *mycobacterium* species which include *Mycobacterium smegmatis, Mycobacterium fortuitum* and *Mycobacterium aurum* (McGaw et al., 2008).

Bioassay guided discovery program

Plants contain a cocktail of many compounds and targeting the bioactive molecule can be a tedious task. To this end, the concept of "bioactivity guided fractionation" was developed to target the isolation of biologically active molecules. Bioassay guided Discovery Program is an inter-disciplinary research between mycobacteriology and natural product chemistry, and this requires a strong collaboration between biologists and chemists. The means of isolation and identification of biologically active compounds from natural sources is referred to as bioassay-guided fractionation (BGF). This methodology involves alternating chromatographic fractionation of extracts and *in vitro* biological testing against a biological target such as the *M. tuberculosis*. Using this method, three potent antimycobacterial compounds were isolated from *Dracaena angustifolia* (Case et al., 2007).

There are advances in fractionation techniques which have been developed towards targeting bioactive compounds in plants. Newer techniques based on liquid-liquid partition, counter-current chromatography (CCC) and centrifugal partition chromatography (CPC), reduce the time-consuming steps experienced with the conventional and older method, adsorption chromatography (Marston and Hostettman, 2006). These techniques have the advantage of loss-free fractionation which is very valuable in bioassay-guided fractionation, as chances of "losing" the anti-TB activity during fractionation are eliminated (Alvi, 2001). There have also been improvements in analytical techniques (fractionation methods hyphenated to spectroscopies or spectometries such as LC-MS, LC-NMR, LC-UV-DAD) to determine structures. Chemical screening of crude extracts using hyphenated techniques allows for the efficient targeted isolation of new types of constituents with potential activities as a complimentary approach to bioassay-guided fractionation (Hostettmann, 1997; Wolfender et al., 2001; Queiroz et al., 2009). The hyphenated NMR technique, which is the most powerful of these, has further advanced in the development of new miniaturized probe technologies, with the aim of increasing sensitivity and reducing costs (Hu et al., 2005). Several workers have successfully employed these hyphenated techniques in the identification and isolation of some compounds (Politi et al, 2004; Fu et al., 2010; Lambert et al., 2007). These methods provide some good preliminary information on the nature of constituents of the extract. With this structural information, once the novelty or utility of a given constituent is established, a scale up of the chromatographic process of fraction can then be done to obtain a good yield of the constituent for full structure elucidation as well as biological and pharmacological testing. In this way, isolation of common compounds of little interest (dereplication) is avoided, saving considerable research time.

Bioassay techniques

Different *in vitro* biological assay methods are used in the biological testing of the efficacy of plant extracts, fractions and compounds against *M. tuberculosis*. These methods include agar disc and well diffusion, micro and macro agar dilution, microbroth dilution, radiorespirometry, reporter gene assays and low oxygen bioassays. Pauli et al. (2005); McGaw et al. (2008) evaluated each of the foregoing methods and discussed their limitations. Results of these bioassays are interpreted as the minimum inhibitory concentration, expressed in terms of µg/mL. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism (Andrews, 2001). The development of gene expression analysis using microarray technology has led to the sequencing of the *M. tuberculosis* genome in 1998 (Cole et al., 1998). This in turn, has facilitated studies on the mechanism of action of antimycobacterial drugs. Transcriptional profiling of both crude extracts and natural products can provide critical information on both mechanism and detoxification that can be used to guide the drug discovery process (Boshoff et al., 2004). This is demonstrated in the DNA microarray analysis carried out on osthole (7-methoxy-8-isopentenoxycoumarin), a coumarin derivative isolated from many medicinal plants such as *Cnidium monnieri* and *Angelica pubescens*, and which had previously shown antimycobacterial activity. Results show that osthole affected a number of important genes involved in

different metabolic pathways in *M. tuberculosis* (Wei et al., 2013).

PLANT NATURAL PRODUCTS AS ANTI-TUBERCULOSIS AGENTS

Worldwide search for plant anti-TB drugs

There has been tremendous research all over the world in the search for novel anti-tuberculosis agents showing that plant derived natural products are potential anti-tuberculosis agents. Research has been performed on screening of plant extracts, based on ethnomedicinal usage, as a preliminary step towards discovering new anti-tuberculosis compounds (Lall and Meyer, 1999; Jimenez-Arellanes et al., 2003; Ibekwe et al., 2012). Despite this, none of the drugs currently used as first or second line drugs in the chemotherapy of tuberculosis has its origin from plant derived natural products. There exist several reports on *in vitro* growth inhibition of different strains of *M*. *tuberculosis* by plant extracts (Newton et al., 2000; Lall and Meyer, 1999; Gautam et al., 2007; McGaw et al., 2008). Some reviews present the different classes of compounds with antimycobacterial activity. Among these are alkaloids, terpenoids, coumarins/chromones, peptides and phenolics (Okunade et al., 2004; Copp, 2003; Copp and Pearce, 2007; Cantrell et al., 2001). A few of these plant metabolites are shown in Table 2.

Plants belonging to different families and genera have shown antimycobacterial activities. It is noteworthy that most of the plants found to be antimycobacterially active, were ethnomedically used for the treatment of tuberculosis or related symptoms such as cough and other respiratory diseases in various societies. Though the compounds responsible for antimycobacterial effects are structurally diverse, they are useful templates for discovery of new pharmaceuticals for the treatment of tuberculosis. Some research has been carried out on structural activity relationships (SAR) by synthesizing derivatives of the parent compounds. This is exemplified in demethoxycurcumin, a compound responsible for the anti-tuberculosis activity of the extracts of *Curcuma longa*. Semi-synthetic modifications of demethoxycurcumin yielded a novel lipophilic analogue, 4-{4-[7-(3-methoxy-4-methylphenyl)-3,5-dioxohepta-1,6-dienyl]-phenoxy}-but-2-enoic acid ethyl ester, which was 25 times more active at 7.8 µg/mL than the parent compound (Table 2). The presence or absence of certain functional groups or moieties has been shown to either increase or decrease bioactivity (Agrawal et al., 2008). With the development of new molecular targeted bioassays such as mycolic acid biosynthesis or cell wall biosynthesis, it will be easier to draw conclusions on structural relationships.

Some Nigerian plants with antimycobacterial activity

Adeleye and co-workers (2008) evaluated the ethanolic and aqueous extracts of 12 Nigerian medicinal plants for antimycobacterial activity. The study revealed that four of the plant extracts (*Allium cepa*, *Allium ascalonicum*, *Terminalia glaucescens* and *Securidaca longepedunculata*) showed activity on both the culture isolate of *M. tuberculosis* and the control strain (*M. tuberculosis* $H_{37}Rv$) at 0.05 mg/mL. Mann et al. (2008) evaluated some Nigerian medicinal plants for antimycobacterial activity and found four plants giving antimycobacterial activity at ≤ 1250 µg/mL. These plants were *Anogeissus leiocarpus*, *Terminalia avicennoides*, *Combretum spp.* and *Capparis brassii*. In another related study, eighty-six Nigerian plant based ethnomedicinal remedies were screened for antimycobacterial activity. Sixty nine percent of the extracts showed anti-tuberculosis activity *in vitro*, with 22% revealing activity at < 500 µg/mL. Some of the plants which showed promising activity were *Ficus sur*, *Pavetta crassipes*, *Combretum molle*, *Waltheria indica* and *Crotolaria lachnosema*, *Anogiessus leiocarpus, Calliandra portoricensis, Cassia sieberiana, Abrus precatorius* and *Cussonia arborea* (Ibekwe et al., unpublished).

NIGERIAN BIODIVERSITY AND CHALLENGES IN TB DRUG DISCOVERY

Nigerian biodiversity, biogeography and anti-TB drug discovery

The biodiversity of the Nigerian flora provides great possibilities in finding novel anti-tuberculosis compounds. Gbile and Adesina (1987), in their review paper, highlighted a good number of Nigerian medicinal plants with biological or therapeutic activities, stressing the pharmaceutical potentials of these plants. Nigeria has a tropical climate with sharp regional variances depending on rainfall. Based on the rainfall distribution, with a wet south and a dry northern half, and also factors such as soil, elevation and human impact on the environment, there are two broad vegetation types; forests and savanna. Nigerian ecology varies from a tropical forest in the south to dry savanna in the far north, yielding a diverse mix of plant and animal life (Microsoft Encarta Encyclopedia, 2006). Every year, millions of square kilometers of biodiversity are lost in Nigeria to the growing herbal market, indiscriminate felling of trees for

Table 2. Some plant anti-TB agents in literature.

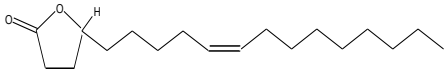
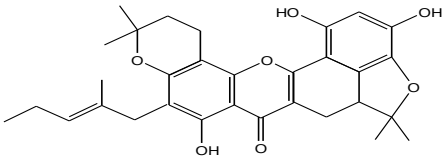
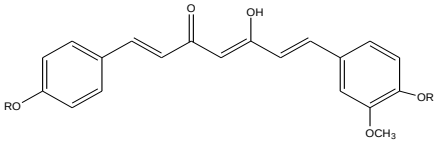
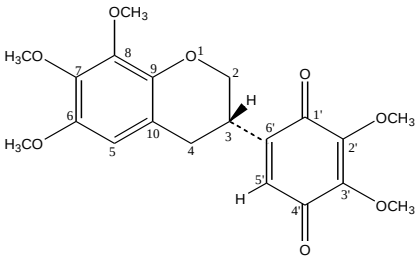
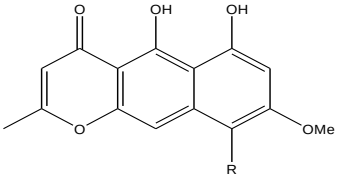
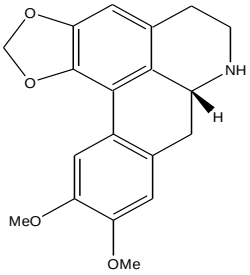
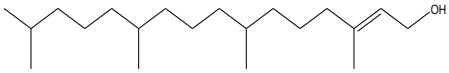
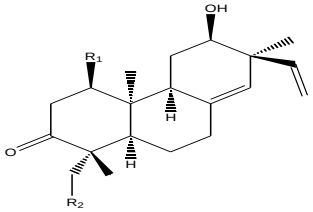
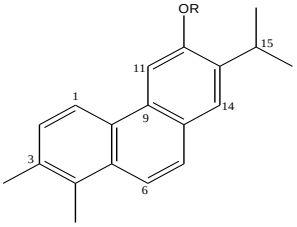
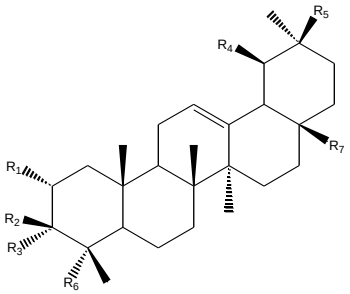
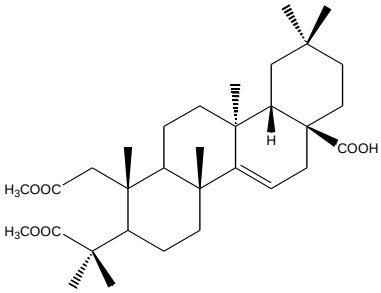
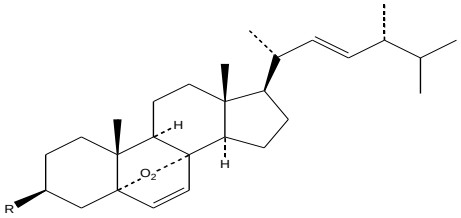
Country	Botanical source	Structure and name of active compound	MIC value (in µg/mL)	Reference
Vietnam	*Micromelum hirsutum*	 (-)-Z-9-octadecenel-4-olide	5.6	Ma et al., 2005
Thailand	*Artocarpus rigidus*	 Artonin F	6.25	Namdaung et al., 2006
India	*Curcuma longa*	 (a) R = H (Demethoxycurcumin) (b) R = CH_2-CH=CH-$COOCH_2CH_3$ (4-{4-[7-(3-methoxy-4-methylphenyl)-3,5-dioxohepta-1,6-dienyl]-phenoxy}-but-2-enoic acid ethyl ester)	(a) 200 (b) 7.8	Agrawal et al., 2008
China	*Abrus precatorius*	 Abruquinone B	12.5	Limmatvapirat et al., 2004
Ecuador and Peru	*Senna oblique*	 (a) R = Me (Quinquangulin) (b) R = H (Rubrofasarin)	12	Graham et al., 2004
Thailand	*Goniothalamus laoticus*	 (-)nordicentrine	12.5	Lekphrom et al., 2009

Table 2. Continue

Taiwan	*Leucas volkensii, Morinda citrifolia, Pourthiaea lucida*	 (E)-phytol	2-32	Rajab et al., 1998; Saludes et al., 2002; Chen et al., 2010
South America	*Sapium haematospermum*	 lecheronol A	4	Woldemichael et al., 2004
Middle East	*Salvia maulticaulis*	 12-demethylmulticauline	0.46	Ulubelen et al., 1997
Latin America, Mexico and South Africa	*Junellia tridens, Valeriana laxiflora, Lantana hispida, Buddleja saligna*	 Oleanolic acid	5- 50	Caldwell et al., 2000, Gu et al., 2004, Jiménez-Arellanes et al., 2007, Bamuamba et al., 2008
Southeast Asia	*Elatoriospermum tapos*	 2,3-secotaraxer-14-ene-2,3,28-trioc acid 2,3-dimethyl ester	4.13	Pattamadilok and Suttisri, 2008
Kenya	*Ajuga remota*	 Ergosterol-5,8-endoperoxide	1	Cantrell et al., 1999

domestic uses, bush burning and urbanization. This loss as predicted on a global basis, will lead to a considerable number of species extinction by the year 2050 (Jenkins, 2003). This effect will undermine the drug discovery process, particularly, bio-conservation in general. The convention on biological diversity (CBD), an international treaty which was ratified in 1992, seeks to promote biodiversity conservation, sustainable use of biodiversity, and equitable sharing of benefits from the use of genetic resources. Though the CBD presents some limitations, especially from the legal angle, as exemplified in a study carried out in Suriname, the overall objective of the treaty should be applauded and abided by (Kingston, 2011).

Nigeria, as a country endemic with tuberculosis, has at its disposal, a huge biodiversity of higher plants and an increasing number of scientists to carry out the needed studies to find plant extracts and compounds active against TB (Cadmus et al., 2010). The country also has a rich culture and history of ethnomedicine. A variety of plants are used locally in the treatment of tuberculosis, but have not been investigated for their anti-tuberculosis properties. These plants provide a rich variety of isolatable phytochemicals, but due to lack of infrastructure and adequate scientific facilities to carry out research, Nigeria and indeed, Africa continues to depend on the Western countries for the discovery of new drugs. Considering the high prevalence of tuberculosis in Africa, it has become imperative for African governments to fund anti-TB drug research and develop capacities more aggressively. The government, pharmaceutical companies and health related non-governmental organizations such as the African Network for Drug and Diagnostic innovation (ANDi) can collaborate in a public private partnership (PPP) scheme to robustly fund research in this area, thus using the biodiversity of the country to address the health needs of the nation, and ultimately promote economic development.

DEDUCTIONS AND CONCLUSIONS

The following deductions and conclusions are evident from the foregoing:

1. Plants are an invaluable source for discovering potentially new antimycobacterial compounds. Positive correlation exists between antimycobacterial activity results on Nigerian plants and ethnomedical/traditional usage.

There is a need for further studies on the correlation between structural features and anti-tuberculosis activity as some bioactive phytochemicals which have proved useful as model compounds or templates can be employed in the synthesis or semi-synthesis of new drugs.

2. Further investigations are needed for the development of new anti-tuberculosis drugs, which include both functional (*in vivo* assays) and mechanistic (micro-array assays) studies. In the future, advances in the understanding of immunology and related areas should permit the development of new selective and sensitive bioassays to guide the isolation of bioactive natural products.

3. If the current trends of destruction of tropical forest habitats and general global simplification of the biota continue at their present rates, biochemists, ethnobotanists, molecular biologists, organic chemists, pharmacognosists, pharmacologists, taxonomists, and other scientists interested and involved in medicinal plant research may have only a few decades remaining to survey and sample the diverse chemical constituents of the plant kingdom for potentially useful novel bioactive compounds. It is imperative that endangered, fragile, and over-exploited genetic resources would be preserved to the greatest extent possible for future generations who may possess the tools (both technical and intellectual) necessary to successfully exploit and manage these resources more intelligently.

4. The government of Nigeria, and indeed, Africa, has a key role to play in the funding of anti-tuberculosis research. With non-profit organisations like ANDi, which is committed to better health care delivery for Africans, by Africans, government can redeem its pledge of the signatories of the 2000 Abuja declaration to increase support for research (including operational research) to develop new tools and improve existing ones.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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The effect of *Agrobacterium* densities and inoculation times on gene transformation efficiency in rubber tree

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Rubber tree belonging to the genus *Hevea* is an economically important crop of Thailand and South-east Asia. To optimize its agronomical trait for glyphosate-resistant, *in vitro* gene transformation through *Agrobacterium tumefaciens* was investigated. The bacteria carrying plasmid pCAMBIA 1304, harboring *gus* as screenable marker genes and *EPSPs* gene was used. The shoot tips were immersed in *A. tumefaciens* suspension at optical densities (OD_{600}) at 0.3, 0.6 and 0.9 for various times (15, 30 and 60 min). The results revealed that shoot explants immersed in *A. tumefaciens* suspension at OD_{600} of 0.6 for 30 min gave the higher survival rate after being cultured on glyphosate containing MS medium for one and half months. Assessment of transformed shoots revealed positive results in GUS histochemical assay. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by polymerase chain reaction (PCR) technique, dot blot hybridization and Southern PCR hybridization. Specific primers for the *gus* and *EPSPs* genes were designed to amplify a 919 and 1,600 bps DNA fragment, respectively.

Key words: Transgenesis, glyphosate, inoculation time, *Agrobacterium* density, *Hevea.*

INTRODUCTION

Hevea brasiliensis Muell. Arg. belongs to the family Euphorbiaceae originated in south America primarily in the Amazon basin, but it is now mainly cultivated in south-east Asia countries. Rubber (cis-1,4-polyisoprene) is created in over 2,000 species of plants distributed along with 300 genera from seven families (Priya et al., 2006), nevertheless there are only one species of plant for the industrial raw material of natural rubber which is *H. brasiliensis*. Natural rubber has high performance properties that cannot be easily replaced by synthetically produced polymers. Consequently, rubber tree is one of the most commercial sources, and the financial records for 42% of the rubber consumed worldwide (Lardet et al., 2011). Conventional breeding and selection method of any crop species, one of the most important parameters, bring together traits of agronomical interest. In case of rubber tree, genetic improvement has been very slow and time-consuming as the major limitations because of narrow genetic base, non-synchronous flowering, low fruit set, long gestation period, heterozygous nature (grafting),

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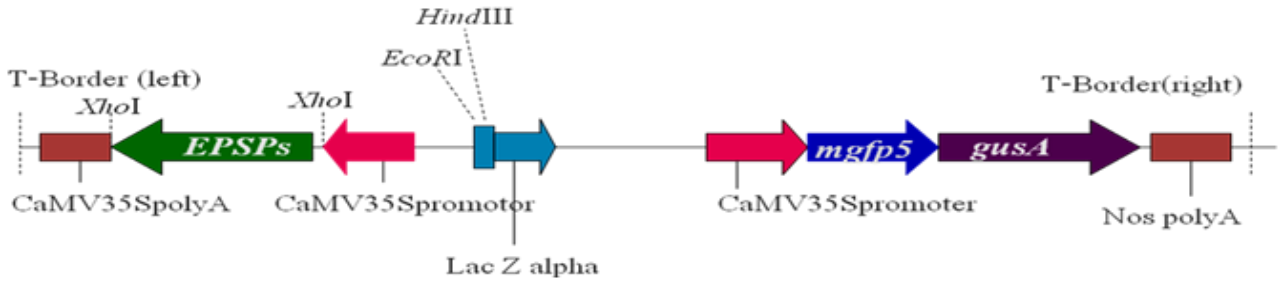

Figure 1. Schematic map of T-DNA region of the binary vector pCAMBIA1304-EPEPs containing the *gus*A gene containing an intron as reporter genes and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance.

insufficient availability of land for field experiments and the absence of fully reliable early selection parameters (Venkatachalam et al., 2007). In addition, breeding program takes up to 25 years for selection and recommend-dation as new clones (Lardet et al., 2011). Its long juvenile phase includes 6-7 years before latex collection.

Genetic transformation offers a potential tool to breeders for adding desirable agronomic traits to crop plants, leading to the development of elite clones in a relatively short period of time (Arokiaraj et al., 2002). *Agrobacterium tumefaciens*-mediated genetic transformation technique has most widely been used for plant species due to its easy protocol without any special equipments. These techniques were developed on numerous *Hevea* clones, GL1, RRII 105 and PB 260 (Arokiaraj et al., 1994; Jayashree et al., 2003; Priya et al., 2006; Montoro et al., 2003; Lardet et al., 2011). Montoro et al. (2003) developed genetic transformation protocol using *A. tumefaciens* in friable integument callus line (clone PB260) for a good frequency of integration of transgenic calli. An efficient genetic transformation procedure was investigated using highly integument-callus lines (Blanc et al., 2006) and GFP selection of transformants (Leclercq et al., 2010).

Nowadays, over-expression of endogenous genes involved in reactive oxygen species scavenging systems, such as MnSOD has been reported (Jayashree et al., 2003). The target of transgene expression in latex cells was also attempted using the promoter from gene *HEV*2.1, which was the major latex Hevein protein (Montoro et al., 2008). Moreover, genetic transformation protocol was developed using the transfer of a synthetic *CP4 EPSPS* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *A. tumefaciens* strain in combination with a novel step-down glyphosate selection system. Glyphosate is a commercial herbicide used in the control of weed species which exerts its action on plants through inhibition of EPSPS.

This chemical is not detoxified, and consequently here is no cross-protection afforded to evolve resistant weeds (Chen et al., 2012).

In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe et al., 2002). However, transformation efficiency was depended on many factors, protocols and cultivar-dependent. Until now there is no report available regarding the gene transformation of glyphosate-resistant shoot of rubber tree.

The introduction of foreign genes into plants was assessed in the transformants by PCR analysis and Southern hybridizations. The process of genetic transformation in *H. brasiliensis* using *A. tumefacients* for glyphosate resistant rubber was optimized.

MATERIALS AND METHODS

Plant material

Seeds from a indigenous clone of rubber tree, naturally grown at Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand, were collected and used as explants for zygotic embryo culture. After 2 weeks of culture, seedlings were obtained and they were excised into two parts, shoot tip and hypocotyl node. The two types of explants were cut into 1.5-2 cm in length and cultured on shoot induction medium (SIM) supplemented with 5 mg/l 6-benzyladenine (BA), 3% sucrose and 0.05% activated charcoal as reported by Te-chato and Muangkaewngam (1992).

The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121°C for 15 min. The cultures were maintained at 28±0.5°C under fluorescent lamps at light intensity of 12.5 µmol/m^2/s; 14 h photoperiod for 1 month. Single shoot at 1 cm was excised and used for transformation.

Bacterial plasmid

A. tumefaciens strain EHA105 containing the plasmid pCAMBIA1304-EPEPs which harbored ß-glucuronidase (*gus*) and 5-enolpyruvylshikimate-3-phosphate synthase (*CP4 EPSPs*) genes (Figure 1) was used in this study. A single colony of this bacteria was pick out and suspended in 25 ml liquid LB medium (10 g/L tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin and incubated on a rotary shaker at 100-150 rpm in the dark at 28°C. After proliferation in LB medium overnight, the cells were collected and resuspended in SIM. The *A. tumefaciens*

suspension was adjusted by spectrophotometer at optical density (OD) of 600 nm and used for transformation.

Agrobacterium densities and inoculation time

The shoot tips were immersed in 25 ml of the *A. tumefaciens* suspension at optical density (OD_{600}) at 0.3, 0.6 and 0.9. The cultures were maintained on rotary shaker in darkness at 100 rpm at 28°C for 15, 30 and 60 min. The explants were placed on sterile tissue papers before transfer to co-cultivation medium which was shoot induction medium (SIM) containing 200 µM acetosyringone. The co-cultivation was kept in the dark at 28°C for 3 days. After co-cultivation, the explants were washed with liquid SIM containing 400 mg/L cefotaxime for 10 min to remove excess bacteria. Then, explants were transferred to SIM supplemented with 200 mg/L cefotaxime to eliminate bacteria for 2 weeks. The inoculated explants were then transferred to selective medium (SIM containing 0.5 mM glyphosate). After 2 weeks of culture, inoculated explants were cultured on SIM containing 2 mM glyphosate for early screening of transformed tissues and subcultured every 2 weeks. The cultures were maintained under 12.5 µmol/m^2/s illumination; 14 h photoperiod at 26±2°C.

Histochemical GUS assay and selection of putative transformants

GUS assays were carried out using protocols described by Jefferson et al. (1987). GUS expression was observed by immersing inoculated explants in X-gluc buffer consisting of 2 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The explants were incubated overnight at 37°C in the dark, and washed with absolute methanol for overnight. The percentage of *gus* expression which was the blue percentage per explant, was recorded and scored under stereomicroscope.

For selection, glyphosate was used for selection of putative transformants. Non-inoculated and inoculated shoots were cultured on selective medium (SIM supplemented with 2 mM glyphosate). After 1 and half months of inoculation, the percentage of glyphosate-resistant shoots [% resistant = (number of survival shoots / total number of shoots) x 100] was recorded.

Molecular analysis of the transformed plantlets by PCR analysis, dot blot hybridization and Southern blot PCR hybridization

Genomic DNA was isolated from young leaf (0.05 g) of non-transformed and transformed plantlets after 1 and half months of culturing on selective medium by the CTAB method (Doyle and Doyle, 1990). The *gus* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3' and reverse primer sequence R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3'. The forward and reverse primer sequences for the *EPSPs* gene amplification were 5'-CCATTCCGCTCGAGATGGCACAAATTAACAACATGGC-3' and 5'-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCGTAT-3', respectively. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2 µl ml of PCR buffer, 0.1 µl of *Taq* DNA polymerase (1 U/ml) which was mixed together and adjusted to 20 µl with sterile distilled water. The PCR reaction started at 96°C for 2 min, followed by 30 cycles of denaturation (96 ^{o}C, 20 s), annealing (55°C, 1 min) and extension (72°C, 2 min), with a final extension of 5 min at 72°C. PCR amplified products were separated in 1.0% agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

For dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (hybond-N, Amersham). Blotted membranes were dried by incubation at 80°C for 1 h. The membranes were pre-hybridized in hybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 1X blocking solution) for 1 h at 65°C. Hybridization was performed with DIG-labeled DNA probe (*gus* or *EPSPs* gene) overnight at 65°C, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridized membrane was washed twice in low stringency buffer (2X SSC, 0.1% SDS) for 15 min, twice in high stringency buffer (0.1X SSC, 0.1% SDS) for 15 min and once in washing buffer (1X maleic acid buffer, 0.3% tween 20) for 10 min. The membrane was blocked in blocking solution (dilute 10X blocking solution 1:10 with maleic acid buffer) for 30 min. After that, the anti-digoxigenin conjugate alkaline phosphate was added into blocking solution and incubated for 30 min. The membrane was then transferred to detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star^{TM}) and exposed to Kodak BiomaX-Omat film for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

For Southern blot PCR hybridization, PCR products 15 µL (1-2 mg/µL) were separated by 1% agarose gel electrophoresis. The gel was treated with 0.25 N HCl to depurinate briefly the DNA and then denatured with an alkaline solution for 30 min and neutralized for 30 min. The denatured DNA was then transferred to a nylon membrane (hybond-N, Amersham). Blotted membrane was dried by incubation at 80°C for 1 h. The blotted membrane was hybridized and detected using the same protocol according to dot blot hybridization as describe above.

Statistical analysis

Data were subjected to ANOVA analysis and significance was determined with the balance analysis test with a level of significance at p = 0.05 using statistically analysis system (SAS). Two factorial completely randomized design with three replicated series was used. Each replication consisted of nine samples.

RESULTS AND DISCUSSION

Agrobacterium densities and inoculation times

The explants immersed in *A. tumefaciens* suspension at optical density of 600 nm at various concentration of *A. tumefaciens* (0.3, 0.6 and 0.9) and inoculation times (15, 30, 60 and 90 min) revealed that shoot explants immersed in *A. tumefaciens* suspension at optical density 600 nm of 0.9 gave the highest GUS expression in all parameters tested. In addition, transient GUS activity increased with inoculation time, reaching 57.67% GUS expression for 90 min, significant difference with the other times of inoculation (Table 1 and Figure 2). In this experiment, the effect of inoculation time on GUS expression was not significantly different. However, the effect of *A. tumefaciens* density was significantly different

Table 1. Effect of *A. tumefaciens* densities and inoculation times on transient expression of the *gus* gene (%) in rubber tree after 4 weeks of transformation.

***Agrobacterium tumefaciens* densities**	**Transient expression of the *gus* gene (%)**			
	Inoculation times			
	15 min	**30 min**	**60 min**	**Mean density**
0.3	35.33^{c}	37.67^{bc}	38.33^{bc}	37.11^{B}
0.6	40.47^{abc}	46.67^{abc}	54.33^{ab}	47.22^{A}
0.9	48.33^{abc}	53.67^{ab}	57.67^{a}	53.22^{A}
Mean time	41.44^{A}	46.00^{A}	50.11^{A}	
CV (%)		20.51		
F (Rep)		1.95 ns		
F (Density)		7.46 *		
F (Time)		2.11^{ns}		
F (Density x Time)		0.28^{ns}		

ns = not significant, *p < 0.05 (significant). Means with different small letter indicate significant differences among treatments (p< 0.05) and mean with different capital letter indicate significant differences among treatment combination. The data are the means from nine samples with three replicates.

in which optical density 600 nm of 0.9 gave the highest GUS expression (53.22%). There was no interaction effect between inoculation times and *A. tumefaciens* densities. Nevertheless, the concentration of *A. tumefaciens* affected survival rate of explants (Figure 3). The result showed that glyphosate-resistant shoots died after 1 and half month of culture on selection medium, which was SIM medium containing 0.5-2 mM glyphosate due to high density of *A. tumefaciens* cell (OD_{600}=0.9). This evident caused an overgrowth of *A. tumefaciens* and decreased in the survival rate of plant tissues. For that reason, the highest glyphosate-resistant shoots were obtained from optical density at 0.6. A similar result has also been reported in many plant species, such as alfafa (Zhang et al., 2010) and *Parthenocissus tricuspidat* (Yang et al., 2010). However, inoculation time was not significantly different in percentage of glyphosate-resistant shoots. Significant interaction was not found between inoculation time and *A. tumefaciens* density in glyphosate-resistant shoots.

In the case of inoculation time, the high transient GUS activity was done from 60 min inoculation time to gene transformation. It showed that shoot explants immersed in *A. tumefaciens* suspension for 90 min gave the highest GUS expression in all treatment tested. However, longer period of inoculation decreased the percentage of survival rate of explants co-cultured with *A. tumefaciens*. According to this result, the inoculation period was critical factor for transformation. The highest survival rate of shoots was obtained from 30 min inoculation (Table 2 and Figure 4). Indeed, the inoculation time of *Agrobacterium* has a close relation with penetration or transmission of T-DNA in the plant tissue. The presence of a larger number of bacterial cells might enhance both the number of transformation events and tissue response related to biotic stress. Kondo et al. (2000) reported that the periods of inoculation seem to be effective for the efficient transfer of the T-DNA into plant cells, and longer periods of inoculation gave negative effect on survival rate of explants. The inoculation time usually applied in transformation procedures is about 30 min for immature embryo of oil palm (Abdullah et al., 2005) and alfafa calli (Zhang et al., 2010), 40 min for embryogenic callus of *P. tricuspidata* (Yang et al., 2010), and up to 2 h for tobacco leaf ring (Vinod Kumar et al., 2005). Interestingly, Blanc et al. (2006) reported that successful in transformation process of rubber tree took only one second submerging calli in *A. tumefaciens* suspension. Contrary results were obtained in the present study. Firstly, different explants type was used. In the present study, shoot explants were applied. Organized tissues seem to resist to *A. tumefaciens* solution better than callus, thus time required for inoculation might be longer. Secondly, regenerability of those explants was far different. Callus of rubber was reported to be very sensitive to all stimulants applied *in vitro*, e.g. toxin, colchicine (Te-chato et al, 1995). Plantlet regeneration from callus just after treating with those chemicals was not reported. Therefore, *A. tumefaciens* desity at optical density 600 nm of 0.6 and inoculation time for 30 min could improve transient GUS expression (46.67%) and glyphosate-resistant shoot (48.67%) for gene transformation procedure in rubber tree.

Molecular analysis of the transformed plantlets by PCR, dot blot hybridization and Southern blot PCR hybridization

To prove the presence of the *gus* and *EPSPs* gene in

Table 2. Effect of *A. tumefaciens* densities and inoculation times on glyphosate resistance shoots (%) of rubber tree after 1 and half months of transformation.

***Agrobacterium tumefaciens* densities**	**Glyphosate resistance shoots (%)**			
	Inoculation times			
	15 min	**30 min**	**60 min**	**Mean density**
0.3	33.33^{bc}	36.33^{abc}	35.67^{acb}	35.11^{B}
0.6	48.33^{a}	48.67^{a}	45.67^{ab}	37.56^{A}
0.9	28.33^{c}	25.67^{c}	26.67^{c}	26.89^{C}
Mean time	36.89^{A}	36.67^{A}	36.00^{A}	
CV (%)		18.99		
F (Rep)		0.15^{ns}		
F (Density)		18.34 *		
F (Time)		0.04^{ns}		
F (Density x Time)		0.18^{ns}		

ns = not significant *p < 0.05 (significant). Means with different small letter indicate significant differences among treatments (p< 0.05) and mean with different capital letter indicate significant differences among treatment combination. The data are the means from nine samples with three replicates.

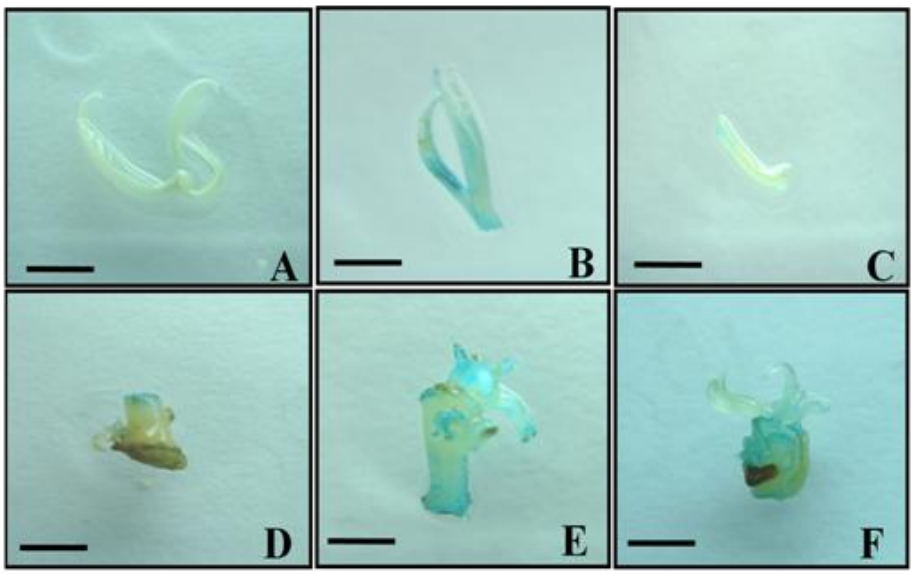

Figure 2. Histochemical assay of β-glucuronidase (GUS) activity in transgenic explants transformed by *Agrobacterium* harboring pCAMBIA1304-EPSPs containing the *gus*A gene and the *EPSPs* gene conferring glyphosate (*N*-phosphonomethyl glycine) resistance (bars= 5 mm). **(A)** Non-transformed leaf. **(B-F)** transformed leaf, petio, stem, shoot and new coming shoot, respectively.

transformed rubber tree, PCR analysis was conducted to evaluate putative transformants, along with non-transgenic plant (negative control). Specific primers for the *gus* gene were designed to amplify a 919 bps DNA fragment. The presence of *gus* genes was confirmed in nine transgenic plants and in the plasmid DNA, whereas the corresponding band was not detected in the non-transgenic control (Figure 5A). For *EPSPs* gene, the transformed plantlets showed the positive results at 1,600 bps of 8 samples from 9 samples (88.89%), but lane number 9 was showed slightly pale band (Figure 5B).

In the case of dot blot hybridization using *gus* gene probe, the genomic DNA of nine samples showed dark color dots indicating the success of gene transfer into plant genome (Figure 6A). On the other hand, dot blot hybridization using *EPSPs* gene showed the positive

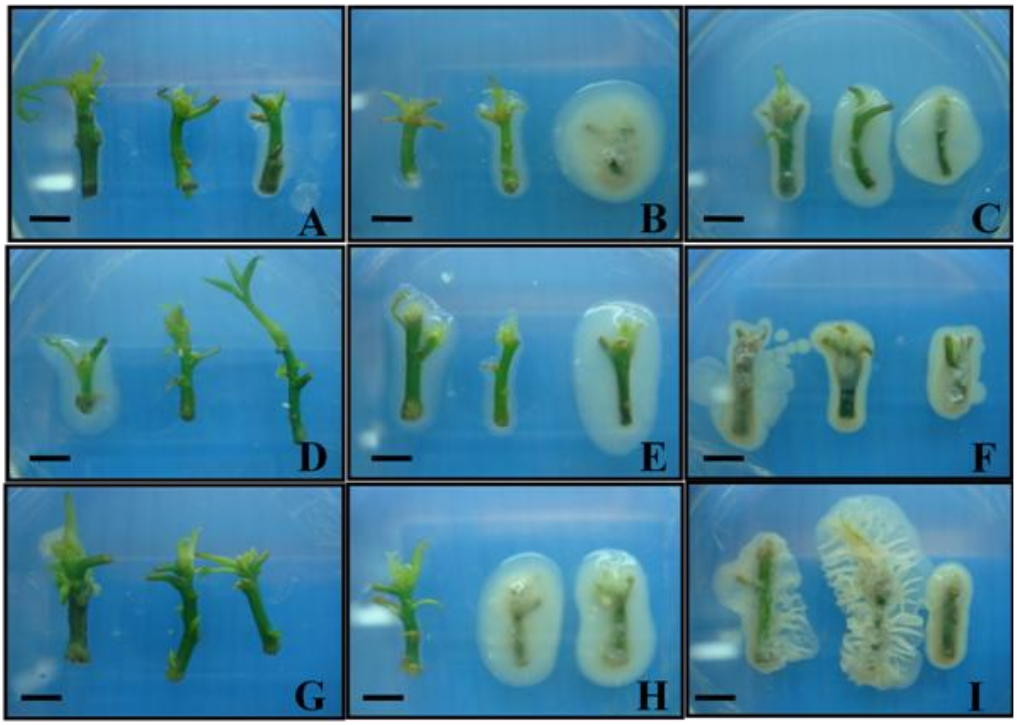

Figure 3. Morphological appearance of shoot explants inoculated with *Agrobacterium* harboring pCAMBIA at various *A. tumefaciens* densities and inoculation times subsequent to culture on co-cultivation medium for 3 days (bars = 5 mm). **(A-C)** Inoculation at OD_{600} = 0.3 for 15, 30 and 60 min, respectively. **(D-F)** Inoculation at OD_{600} = 0.6 for 15, 30 and 60 min, respectively. **(G-I)** Inoculation at OD_{600} = 0.9 for 15, 30 and 60 min, respectively.

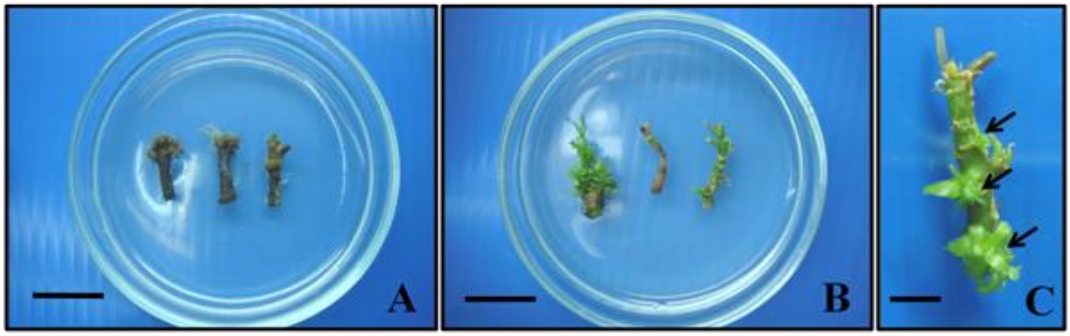

Figure 4. Glyphosate-resistant shoots cultured on selective medium (bar=1 cm). **(A)** Control (non-transformed shoot). **(B)** Shoot inoculated *A. tumefaciens* with OD_{600} = 0.6 for 30 min. **(C)** Newly shoot after culture on selection medium for 1 and haft months.

results of 7 samples from 9 samples (77.78%). The positive transgenic plant samples developed dark black spots as well as the positive control sample, while the non-transformed plantlet samples did not show the dark spots (Figure 6B).

Southern PCR hybridization clearly confirmed the presence of *gus* gene and *EPSPs* gene at sizes 919 and 1,600 bps, respectively. The band of DNA from non-transformed shoot did not appear (Figure 7C). However, Southern PCR hybridization using *EPSPs* gene gave the same result in dot blot hybridization. Only seven positive transgenic plant samples developed dark black bands as well as the positive control samples, while 2 samples and the non-transformed samples did not show the dark bands (Figure 7D). The reason might be due to the transportation of T-DNA from *Agrobacterium* to plant

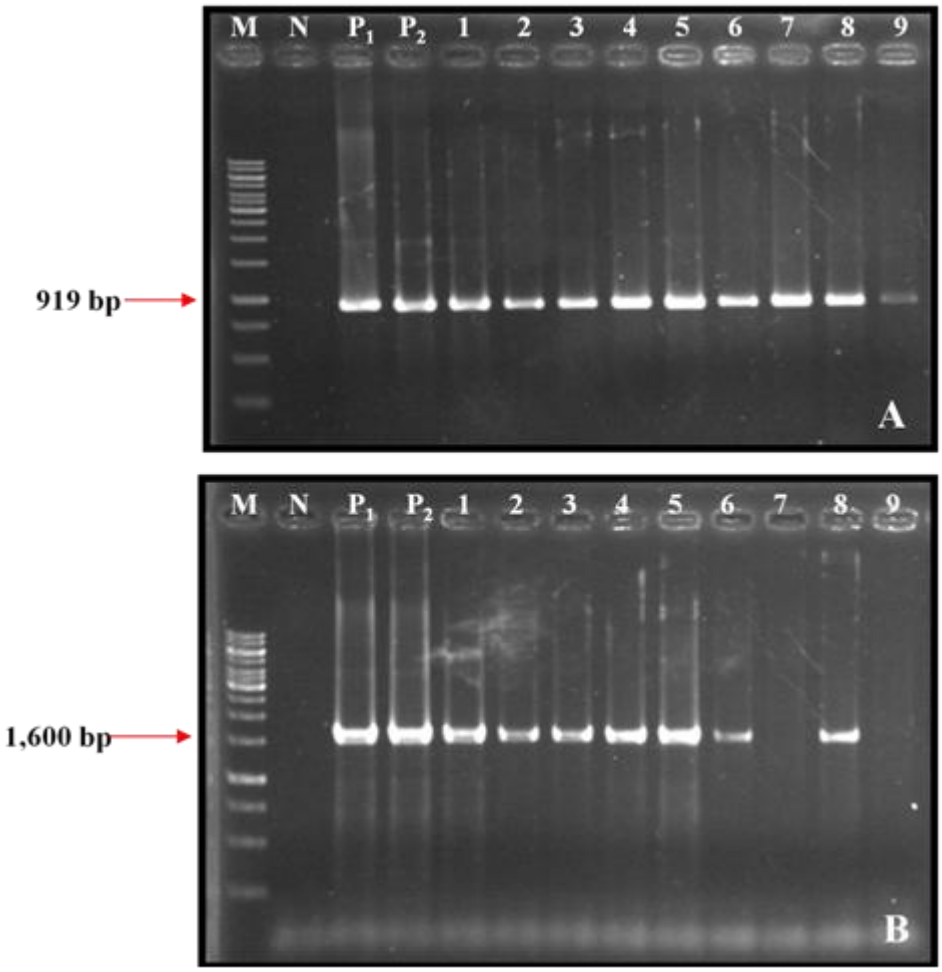

Figure 5. PCR analysis showed the presence of **(A)** *gus* gene at 919 bps and **(B)** *EPSPs* gene at 1,600 bps from different plant genomes. M, marker; N, negative control; P, positive DNA control; 1-9, transformed shoots.

Figure 6. Detection of (A) *gus* gene and (B) *EPSPs* gene in genomic DNA of transformed shoots after 1 and half months on selection medium by dot blot hybridization. P, positive control; N, non-transformed; 1-9, transformed shoots.

genome. Enzyme endonuclease cut T-DNA at right border (RB) from Ti plasmid and inserted RB border into plant genome before left border (LB). Right border connected with *gus* and *EPSPs* genes, respectively. In some case, it is possible that incomplete transfer of T-DNA was performed. For this result, only some reporter genes at the first part was sent to plant genomes while the others were not. Thus, in the present study, all of the transgenic samples presented the *gus* gene, but *EPSPs* gene did not show in some samples.

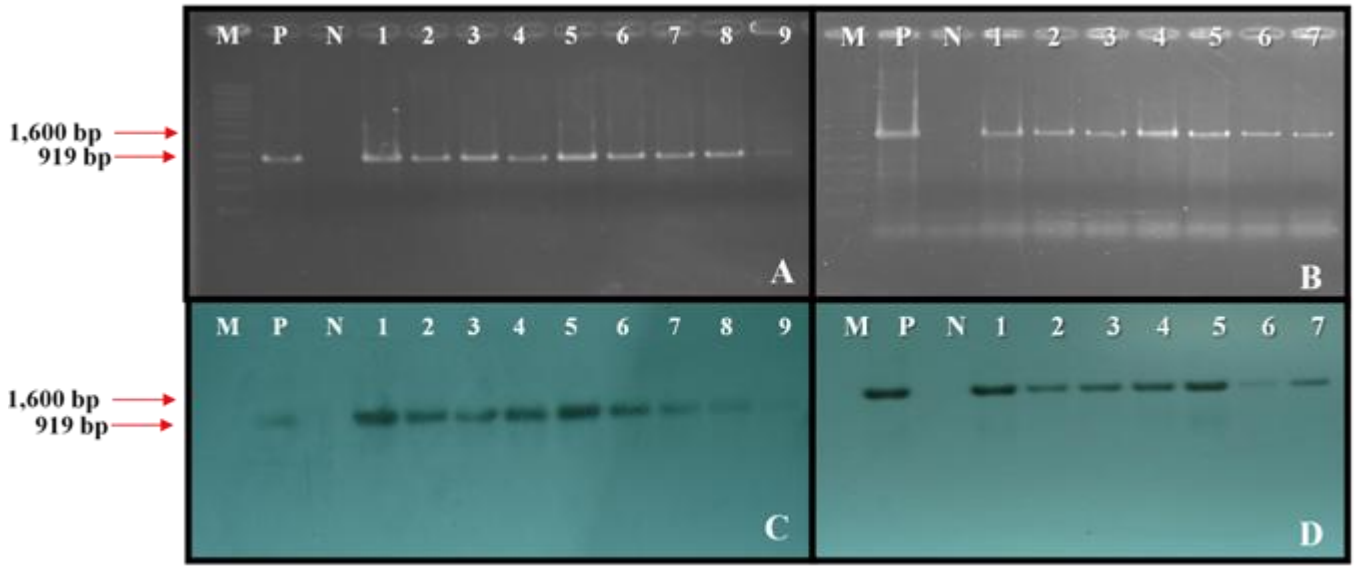

Figure 7. Detection of **(A, C)** *gus* gene and **(B, D)** *EPSPs* gene in transformed shoots after 1 and half months on selection medium by PCR **(A, B)** and Southern PCR hybridization **(C, D).** M, DNA marker; P, positive control; C, non-transformed; N, negative control; 1-8, transformed shoots.

Conclusion

Shoot tip explants inoculated with *A. tumefaciens* carrying plasmid pCAMBIA 1304, harboring *gus* gene as screenable marker genes and *EPESPs* gene for 30 min at optical density of 600 nm to 0.6 gave the highest survival rate of the explants. GUS histochemical assay of transformant revealed positive results. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by PCR technique dot blot hybridization and Southern PCR hybridization. Specific fragments of DNA at size of 919 and 1600 bps conferred glucuronidase gene and glyphosate resistance were identified by those techniques.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Does foliar application of salicylic acid protects nitrate reductase and enhances resistance in virus infected maize?

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The present study was conducted to assess whether exogenous applied salicylic acid (SA) as a foliar spray could ameliorate the adverse effects of virus infection in two maize cultivars (maize cv. sabaini and maize cv. Nab El-gamal). The plants were grown under normal field conditions for two weeks in sand clay soil, and then sprayed with either 2 or 4 mM SA. Two weeks later, plants were subjected to infection with two different concentrations of virus (TMV1 and TMV2), and were harvested 10 days later. Fresh and dry matter, shoot and root lengths, proline, soluble protein, soluble sugars as well as nitrate reductase activity were measured. Both fresh and dry matter were decreased under virus infection however, SA enhanced the fresh and dry matter production in both cultivars regardless the type of virus or SA concentration used. In roots, both fresh and dry matters were not affected. The shoot length was enhanced by salicylic acid than root length regardless the concentration used or virus treatment. The water content was much higher in shoots than roots especially in maize cultivar sabaini. Proline was accumulated in SA virus infected plants than reference control especially in cv sabaini. Soluble proteins and soluble sugars were accumulated in SA virus infected plants and in cv sabaini more than Nab El-gamal as compared with reference control. NRA was reduced in virus infected cultivars and cv sabaini was dramatically affected than Nab El-gamal. Treatment of plants with SA had a positive effect on preserving the activity of NR but was still less than the reference control regardless the cultivar used.

Key words: Nitrate reductase, proline, protein, salicylic acid, sugars, viruses.

INTRODUCTION

Salicylic acid (SA) is a component of the signal transduction pathway needed for induction of systemic acquired resistance, a plant-wide enhancement of resistance against a broad spectrum of pathogens (Murphy et al., 2001).

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Abbreviations: SA, Salicylic acid; **SAR,** systemic acquired resistance; **FW,** fresh weight; **DW,** dry weight; **NRA,** nitrate reductase activity; **TMV,** tobacco mosaic virus.

The trigger for SA synthesis and induction of systemic acquired resistance is the recognition of an invading microorganism by a product of a resistance gene (Baker, 1997). Often, this recognition is accompanied by the hypersensitive response a form of rapid programmed host cell death in a region around the point of pathogen entry (Hammond-kosack and Jones, 1996).

Salicylic acid, which is naturally synthesized by plants, plays an important role as a signal molecule that induces the tolerance mechanisms under the influence of both biotic and abiotic stresses such as virus, bacteria, fungi infections, freezing, drought, heat and heavy metals (Yalpani et al., 1994; Dat et al., 1998 Senaratna et al., 2000, Hussain et al., 2011). When plants are exposed to salt stress, they adapt their metabolism in order to cope with the changed environment.

Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals and instigate biochemical changes that adjust the metabolism accordingly (Hasegawa et al., 2000). Moreover, under salt stress, SA applications were found to enhance the biosynthesis of proline, photosynthetic pigments, enzymatic and non-enzymatic antioxidants which all are stated as related to plant stress tolerance (Shakirova et al., 2003). Several studies also supported a major role of SA in modulating the plant response to several a biotic stresses including salt and water stress (Yalpani et al., 1994; Senaratna et al., 2000). In maize plants, pre-treatment with SA induced the production of antioxidant enzymes, which in turn increased chilling and salt tolerance (Janda et al., 1999). The objective of the present study was to assess the role of exogenous salicylic acid applications for tolerance in maize subjected to virus infection as well as to investigate the effects of SA individually and accompanied with virus infection on two maize cultivars.

MATERIALS AND METHODS

Two maize cultivars (maize Sabaini and Nab El-gamal) were brought from agronomy department, Faculty of Agriculture, Minia University, El Minia, Egypt. The kernels were left to germinate in the following manner: 1) Group A without any treatment (virus or salicylic reference control); 2) Group B treated with virus TMV1 (50%); 3) Group C treated with virus TMV2 (75%); 4) Group D combination of TMV1 + 2 mM salicylic; 5) Group E combination of TMV2 + 2 mM salicylic; 6) Group F combination of TMV1 + 4 mM salicylic, and 7) Group G combination of TMV2 + 4 mM salicylic.

Kernels of two maize cultivars were germinated in pots with sand clay soil at normal field conditions. Plants were left to grow for two weeks and then sprayed by salicylic acid (2 or 4 mM). Two weeks later plants were also treated with viruses (TMV, 50 and 75% 100 µl each) by making injurious infections in leaves by carborandum (10 days later plants were harvested). To determine the dry matter, the freshly harvested organs (shoots and roots) were dried in an aerated oven at 105°C for 24 h. The soluble proteins were determined according to the method adopted by Lowery et al. (1951). Free proline was determined according to Bates et al. (1973), soluble sugars by anthrone suphuric acid method which was carried out by Fales (1951) and Schlegel (1956) and adopted by Badour (1959).

NR-activity

Leaves (1 g fresh weight) were ground with mortar and pestle in liquid nitrogen. Two (2) ml extraction buffer (100 mM Hepes-KOH pH 7.6; 20 mM $MgCl_2$, 10 µM FAD, 5 mM DTT, 1 mM Pefabbloc, 0.2 mM PMSF, 1% polyvinyl pyrrolidone (PVP) and 0.05% casein) were added to the still frozen powder and grinding continued to thaw. The suspension was then centrifuged for 12 min (4°C, 12000 rpm) and the supernatant was removed and kept on ice. The reaction medium contained (total volume 1 ml) 50 mM HEPE pH 7.6, 10 µM FAD, 1 mM DTT, 5 mM KNO_3, 0.2 mM NADH and either 20 mM $MgCl_2$ or 20 mM EDTA. The reaction was started by addition of 100 µl extract and terminated after 5 min by addition of 125 µl zinc acetate solution (0.5M). After a short centrifugation (4°C, 5 min, 12000 rpm), 10 µl PMS was added to 950 µl of the supernatant in order to oxidize excess NADH. After 20 min in the dark, formed nitrite was measured colorimetrically by adding 750 µl of 1% sulfanilamide in 3 M HCl, and 750 µl of 0.02% N-naphthyl-ethylene diamine hydrochloride, and absorption was determined at 546 nm. For each series, blank and a nitrite standard (20 µM KNO_2) was included.

The data of all experiments were subjected to one way analysis variance and means were compared using the least significant difference test (L.S.D.) using statistical program (Sta. Base. Exe.) on computer.

RESULTS

Salicylic acid is an endogenous growth regulator of phenolic nature, which participate in the regulation of physiological processes in plants such as growth, photosynthesis, nitrate metabolism and also provide protection against biotic and a biotic stresses. The use of salicylic acid (2 and 4 mM) had different effects on both maize cultivars treated with different viruses (TMV1 and TMV2) during the vegetative growth. In maize Nab El-gamel, both fresh and dry weight of plants were not affected by virus TMV1, however treatments with TMV2 enhanced both fresh and dry matter over control by 107.2 and 107.6%, respectively (Table 1). The pretreatment of plants with salicylic acid 2 or 4 mM had a significant effect on both fresh and dry matter especially plants treated with 4 mM salicylic acid and TMV1 which have 151.1 and 131.5% fresh and dry matter, respectively of virus treated plants.

In roots, both fresh and dry matter were not affected with both treatments (salicylic or viruses) except for 2 mM SA treated with TMV1 which had 125.5 and 120% of virus treated plants only (Table 1). The shoot length was significantly enhanced by salicylic acid more than root length regardless the concentration of SA used or virus treated. The relative water content of both shoots and roots of maize cultivar was increased in plants treated with salicylic and infected with viruses regardless the concentration used or virus treated (Table 1). The highest values of relative water content were obtained (88.4 and 82.9% of control plants, respectively) in both shoots and

Table 1. Effect of virus treatment on fresh and dry matter (gm), relative water content (RWC) shoot and root lengths (cm) of maize Nab El-gamal treated with different concentrations of SA.

Treatment	Shoot				Root			
	FW	DW	RWC	Length	FW	DW	RWC	Length
Reference	9.6 ± 0.32	1.3 ± 0.1	86.4	45.2 ± 1.1	2.03 ± 1.1	0.43 ± 0.3	78.8	20 ± 9.5
V 1	9.8 ± 4.8	1.3 ± 0.6	86.7	49.6 ± 5.6	1.96 ± 0.8	0.40 ± 0.3	79.5	17.6 ± 3
V2	10.3 ± 1.0	1.4 ± 0.1	86.4	46.2 ± 1.8	2.0 ± 0.4	0.52 ± 0.05	74.0	18.3 ± 6
V1+2 mM SA	14.1 ± 4.7	1.7 ± 0.6	87.9	53 ± 3.8	2.46 ± 0.4	0.48 ± 0.02	80.5	19.8 ± 3
V2+ 2 mM SA	11.3 ± 2.2	1.41 ± 0.4	87.6	43.9 ± 0.6	1.66 ± 0.1	0.41 ± 0.02	75.3	15.3 ± 3
V1+ 4 mM SA	14.8 ± 3.2	1.71 ± 0.4	88.4	49.6 ± 6.3	1.86 ± 0.6	0.42 ± 0.1	77.4	19.2 ± 2.8
V2 +4 mMSA	13.8 ± 2.6	1.75 ± 0.5	87.3	45.2 ± 2.3	2.1 ± 1.0	0.36 ± 0.3	82.9	16.5 ± 4.1
LSD at 5%	3.9	0.52		8.4	1.16	0.19		6.12

RWC, Relative water content; FW, fresh weight; DW, dry weight. Data means of 3 replications ±SD.

Table 2. Effect of virus treatment on fresh and dry matter (gm), relative water content (RWC) shoot and root lengths (cm) of maize Sabaini treated with different concentrations of SA.

Treatment	Shoot				Root			
	FW	DW	RWC	Length	FW	DW	RWC	Length
Reference	8.3 ± 1.8	1.3 ± 0.32	84.3	38.7 ± 6.0	2.5 ± 1.3	0.38 ± 0.08	84.8	16.7 ± 0.7
V 1	9.03 ± 3.4	1.33 ± 0.2	85.3	37.8 ± 5.6	2.4 ± 0.78	0.44 ± 0.17	81.7	18.7 ± 8.0
V2	8.2 ± 2.8	1.1 ± 0.37	86.6	38.9 ± 9.5	1.5 ± 0.44	0.25 ± 0.01	83.3	17.4 ± 3.2
V1+2 mM SA	6.3 ± 1.2	0.92 ± 0.2	85.4	34 ± 3.1	1.7 ± 0.6	0.26 ± 0.12	84.7	18.4 ± 2.7
V2+ 2 mM SA	7.7 ± 2.3	1.1 ± 0.3	85.7	38.8 ± 5.1	1.8 ± 0.3	0.26 ± 0.04	85.3	14.6 ± 2.0
V1+ 4 mM SA	9.9 ± 2.4	1.31 ± 0.4	86.9	39.9 ± 2.3	1.66 ± 0.7	0.28 ± 0.03	83.1	18.1 ± 1.3
V2 +4 mM SA	7.9 ± 2.9	1.1 ± 0.3	86.1	34.9 ± 6.2	3.2 ± 1.1	0.39 ± 0.4	87.8	17.8 ± 2.1
LSD at 5%	3.14	0.38		7.82	1.33	0.20		6.12

Data means of 3 replications ±SD.

roots.

In maize cultivar Sabaini, the plants responded differrently to both salicylic and virus infection compared with cultivar Nab El-gamal. Both fresh and dry matter of shoots and roots were slightly enhanced by salicylic acid (Table 2). The most obvious increase in fresh and dry matter over control plants were obtained with V1+4 mM SA (119.2 and 100.7%, respectively). However, in roots, the most enhanced effect of salicylic acid was obtained with V2+4 mM SA (128 and 102.6%) of control plants. Both shoot and root lengths were slightly affected by both treatments regardless the virus or salicylic acid used. The values of water content were obvious in roots than shoots in all treatments; the highest water content was obtained with virus and 4 mM SA (86.1 and 87.8%) in both shoots and roots (Table 2).

Plants adapt to stress by changes in cellular metabolism. A number of these adaptive responses are associated with the accumulation of osmolytes like proline and sugars. The proline concentration in maize cultivar Nab El-gamal was increased under virus treatment compared with control (Table 3). The treatment of maize with exogenous application of SA had a marked and significant effect on proline accumulation in both shoots and roots. It is worth to mention that, shoots accumulated proline more than roots. Maize sabaini (shoots) accumulated proline around two folds higher than reference control (153.5%), however in roots; proline was decreased with salicylic acid treatment.

The soluble proteins were markedly increased in both maize cultivars regardless the organ analyzed or treatment used (Table 4). It is worth to mention that, plants infected with virus and treated with 4 mM SA significantly enhanced the accumulation of soluble proteins about two folds higher than reference control (273.3 and 260%) in shoots of both cultivars.

Virus infection reduced nitrate reductase activity in shoots of both maize cultivars (Table 5). The reduction was obvious in maize Nab El-gamal than maize sabaini. The activity of nitrate reductase in maize sabaini was sensitive compared to maize Nab El-gamal. The treatment with any of the two viruses' concentrations resulted in reduction of activity. SA treatments had a protective effect on nitrate reductase activity but the

Table 3. Effect of virus treatment on proline concentration (mg/gDW) for both maize cultivars (Nab El-gamal and Sabaini) treated with different concentrations of SA.

Treatment	Nab El-gamel		Sabaini	
	Shoot	Root	Shoot	Root
Reference	0.21 ± 0.02	0.08 ± 0.02	0.28 ± 0.05	0.12 ± 0.01
V 1	0.23 ± 0.01	0.19 ± 0.02	0.15 ± 0.01	0.15 ± 0.02
V2	0.26 ± 0.04	0.09 ± 0.04	0.42 ± 0.01	0.2 ± 0.02
V1+2 mM SA	0.27 ± 0.01	0.15 ± 0.02	0.27 ± 0.01	0.14 ± 0.01
V2+ 2 mM SA	0.24 ± 0.03	0.09 ± 0.03	0.32 ± 0.05	0.06 ± 0.01
V1+ 4 mM SA	0.24 ± 0.02	0.12 ± 0.03	0.24 ± 0.04	0.07 ± 0.01
V2 + 4 mM SA	0.25 ± 0.02	0.08 ± 0.02	0.43 ± 0.05	0.08 ± 0.01
LSD at 5%	0.044	0.047	0.064	0.027

Data means of 3 replications ±SD.

Table 4. Effect of virus treatment on soluble protein concentration (mg/g DW) for both maize cultivars (Nab El-gamal and Sabaini) treated with different concentrations of SA.

Treatment	Nab El-gamel		Sabaini	
	Shoot	Root	Shoot	Root
Reference	10.9 ± 1.6	12.8 ± 0.98	20.9 ± 2.8	17.6 ± 0.91
V 1	13.2 ± 1.3	11.7 ± 0.7	19.9 ± 1.1	41.4 ± 5.8
V2	19.9 ± 1.1	10.9 ± 0.98	23.3 ± 1.7	16.1 ± 0.96
V1+2 mM SA	13.7 ± 0.63	20.5 ± 3.1	40.8 ± 3.7	13.3 ± 1.2
V2+ 2 mM SA	17.43 ± 1.8	19.3 ± 1.1	36.5 ± 1.8	32.7 ± 0.84
V1+ 4 mM SA	13.7 ± 0.6	28.5 ± 4.5	54.5 ± 2.4	30.3 ± 2.8
V2 + 4 mM A	29.8 ± 1.1	7.7 ± 0.8	34.5 ± 2.6	26.5 ± 1.7
LSD at 5%	2.08	3.17	4.20	4.43

Data means of 3 replications ±SD.

Table 5. NR activity (µmol g^{-1} FW h^{-1}) and soluble sugars (mg/g Dw) in shoots of both maize cultivars infected with virus and treated with different concentrations of SA.

Treatment	Nab el- gamal		Sabaini	
	Shoot NR	Soluble sugars	Shoot NR	Soluble sugars
Reference (NRA)	15.6 ± 3.2	29.8 ± 3.2	6.8 ± 0.35	32.8 ± 3.3
V 1 (NRA)	7.5 ± 0.8	36.3 ± 4.2	4.9 ± 0.7	38.7 ± 4.4
V2 (NRA)	4.2 ± 0.5	35.4 ± 2.5	3.8 ± 0.4	39.4 ± 2.8
V1+2 mM SA (NRA)	9.9 ± 0.2	45.2 ± 1.8	4.6 ± 0.8	59.2 ± 5.1
V2+ 2 mM SA (NRA)	8.4 ± 0.5	42.5 ± 4.2	4.4 ± 0.23	55.4 ± 3.2
V1+ 4 mM SA (NRA)	7.5 ± 0.9	31.6 ± 5.4	3.9 ± 0.5	44.6 ± 4.3
V2 + 4 mM SA (NRA)	8.7 ± 0.3	33.8 ± 3.3	4.2 ± 0.9	46.3 ± 7.1
LSD at 5%	1.3	5.02	1.11	7.95

Data means of 3 replications ±SD.

activity still lower than reference control.

DISCUSSION

Maize is widely cultivated throughout the world and a greater weight of maize is produced each year than any other grain. The mitigation effect of SA to a biotic stress was investigated through SA application by foliar spray of maize (Khodary, 2004). For last two decades, SA has received much attention because of its involvement in plant defense mechanisms against both biotic and abiotic stresses. Biotic stresses have been demonstrated recently; Sakhanokho and Kelley (2009) recorded that SA

typically showed the salt tolerance under *in vivo* conditions in two botanical medicinal *Hibiscus* species. SA functions in plants as a key component of the signal transduction pathway leading to the induction of SAR and plays a role in resistance to all microbial pathogens, including fungi, bacteria, and viruses (Delaney et al., 1994).

From the results of the present study, it is obvious that virus treatments decreased the fresh and dry matter of both maize cultivars. The reduction was pronounced in maize sabaini than maize Nab El-gamal. However, exogenous application of SA often improved the plant growth in virus infected plants in both cultivars. Many studies supports that SA induced resistance of maize to salinity and osmotic stress (Tuna et al., 2007) and in wheat (Mutlu et al., 2009). Exogenous application of SA stimulated N and relative water content (Shirasu,et al., 1997) and this is in accordance with our results. SA-induced increase in growth could be related to enhancement of antioxidants that protect the plants from oxidative damage El-Tayeb (2005) or enhanced photosynthetic capacity in maize (Khan et al., 2003). The increase in fresh weight and water content under SA treatment in our results could be attributed to the conservation of plants to water under virus and SA treatments (Barkosky and Einhelling, 1993). The observed increase in plant height, shoot biomass and in root biomass in both maize cultivars treated with SA may be related to the ability of SA to induce antioxidant responses that protect them from damaging (Senaratna et al., 2000).

Accumulation of proline is a mechanism by which plants adapt to water stress and create anti stress defense. Plants that produce higher levels of proline are able to survive under stress (Delauney and Verma 1993; Szepesi et al., 2005). The protective action of SA during virus stress was demonstrated by enhanced proline production particularly in shoots of maize sabaini treated with (V2+4 mM SA) which reached ~153.3% of control plants.

The production of soluble proteins under virus infection in both maize cultivars especially cultivar sabaini treated with (V1+4 mM SA) which reached around (260.7%) of control plants, supported by increased relative water content (RWC) in that cultivar, proved that the accumulation of osmolytes including sugars also allows additional water to be taken up from environment which reduce water shortage within the plants and help to stabilize protein structure (Low, 1985). This is in accordance with the results obtained by Zahra et al. (2010) working with tomato with observed increase in leaf protein levels.

Nitrate reductase (NR, EC1.6.6.1) is localized mainly in the cytosol and its expression at the transcriptional levels is affected by nitrate, light and plant hormones. Stress provokes either increase, decrease as well as no effect on nitrate reductase activity (Abd El- Baki et al., 2000). Virus treatments inhibited nitrate reductase activity in both maize cultivars. The activity of NR was lower in maize sabini than maize Nab El-gamal, however the drop in activity in sabaini under virus stress was lower than Nab El-gamal. An efficient N assimilation is said to be favored by a high rate of CO_2 assimilation (Ferrario et al., 1998). SA induced conservation of water in stressed plants and also accumulated sugars which favored NR activity and protection in SA virus treated plants, this probably reflects the maintenance or even induction of root elongation at virus infected plants, which can be considered as an adaptive response to stress (Balibrea et al., 2000). The induction of NR activity may be also due to liberation of nitrate from vacuole under SA which favor NR activity since nitrate affects NR- mRNA (Abd El- Baki et al., 2000).

In conclusion, SA may probably induced resistance to TMV infected maize in large part by inhibiting virus replication (Chivasa et al., 1997) or resist cell to cell movement. We also conclude that cell and tissue development exerts a powerful influence over the design of the defensive signaling pathway and the resistance mechanisms that they trigger. The future application of this plant hormone holds a great promise as a management tool for providing tolerance to our agricultural crops against stress agents (biotic or abiotic) aiding to improve crop yield in near future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effect of different growth parameters on chitinase enzyme activity of acridine orange and ethidium bromide mutant bacteria of the gut environment

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The degradation of chitin is mediated primarily by bacterial chitinases. Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitinases have shown numerous applications in waste treatment and management of shellfish processing industries. Therefore, in the present study, an attempt was made to optimize chitinase production by one of the shrimp's gut mutant bacterial flora, *Pseudomonas alcaligenes*. Optimization of culture conditions revealed that the enzyme production was maximum in pH 7.5 (107.4 ± 0.50 U/ml), temperature 35°C (103.15 ± 1.74 U/ml) when the carbon and the nitrogen sources used were CMC (106.0 ± 1.89 U/ml) and KNO_3 (91.2 ± 1.51 U/ml), respectively. The total chitinase production for all optimum conditions is 105.07 ± 1.33 U/ml.

Key words: Chitinase, shrimp gut, mutant, *Pseudomonas alcaligenes*, optimization condition.

INTRODUCTION

Chitin, a homopolymer of β-1, 4-N-acetyl-D-glucosamine (Glc NAc), is one of the most abundant natural polymers. Recycling of chitin from disposed materials and dead organisms result mainly from the activity of chitinolytic microorganisms (Brurberg et al., 2000; Jindra et al., 2001). Species of the genera *Bacillus, Serratia* and *Vibrio* have been reported to secrete several chitinolytic enzymes and chitin binding proteins, which are thought to degrade chitin synergistically into the extracellular environment (Amit Kumar et al., 2007). Quantitative enhancement of enzyme over production by bacteria requires strain improvement as the quantities produced by wild strains are usually too low (Bapiraju et al., 2004). Several strains of microorganisms have been selected or genetically modified to increase the efficiency with which they produce enzymes (Okonko et al., 2006).

Chemical agents known to be effective in increasing the rate of mutations in higher organisms have similar

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effects on bacteria. The mutations induced by these agents in bacteria, as in higher organisms, seem to be random and non specific (Witkin, 1946). Considering the paucity of information available on enzyme production by wild and mutant microflora of shrimps gut environment, an attempt was made in the present study to isolate and to identify them and to screen their chitinolytic activity and to examine their enzyme producing ability with varying nutritional sources, pH and temperature.

MATERIALS AND METHODS

Penaeus monodon which inhabits in marine forms and is endemic in Peninsular India and other countries is an important marine crustacean. It was collected from the Rajakkamangalam estuary at Rajakkamangalam, Kanyakumari District, Tamilnadu. The collected shrimps were aseptically transferred to the laboratory for further study.

Isolation, identification and screening of gut bacterial flora

The weight of shrimp was noted and the gut was aseptically dissected out and serially diluted upto 10^{-5} dilution. From each dilution, 0.1 ml of sample was taken and spread on nutrient agar. The plates were incubated at 37°C for 24 to 48 h and the total viable counts (TVC) of colonies were finally noted. The isolated cultures were purified individually by streaking on nutrient agar and were subcultured. Then, the bacterial cultures were identified by performing biochemical tests.

Chitinase activity

The chitinase detection agar (CHDA) (components (g/l) colloidal chitin, 10.0 g; agar, 20.0 g; soya bean powder, 20.0 g; starch, 3.0 g; peptone, 3.0 g; yeast extract, 2.0 g; $CaCO_3$, 1.0 g; M9 medium, Na_2HPO_4, 0.65 g; KH_2PO_4, 1.5 g; NaCl, 0.25 g; NH_4Cl, 0.5 g; $MgSO_4$, 0.12 g; $CaCl_2$: 0.005 g; pH : 6.5) plates were prepared. The isolated gut microbes were single streaked individually into the CHDA plates and were incubated at 37°C for 72 h. They were observed for zone formation. The colonies which formed a zone around them were chitinase positive strains, which were sub cultured regularly for further study.

Preparation of colloidal chitin (2%) (Roberts and Selitrennikoff, 1988)

Twenty gram of chitin powder was added into 180 ml of 37% HCl under vigorous stirring for 2 h. It was poured into 1 L of ice cold ethanol (95%) under vigorous stirring for 30 min. This suspension was stored at 20°C until further use. When in need, 10 ml of the suspension was centrifuged at 5,000 rpm for 15 min. The precipitate was collected and washed with 50 ml of 50 mM sodium acetate buffer (pH 6.8). The above process was repeated 3 times and the precipitate derived was dissolved in 90 ml of 50 mM sodium acetate buffer (pH 6.8). This was the prepared 2% colloidal chitin.

Chemical mutation

The test organisms were inoculated into the nutrient broth and incubated at 37°C for 24 h. After incubation, the organisms were incubated at 37°C for 24 h. After incubation, the organisms were inoculated into Luria Bertani broth and incubated at 37°C for 24 h. The above cultures were serially diluted on normal saline solution and the 10^{-2} dilution was selected and plated into LB agar plates with different chemical mutagens such as ethidium bromide (200 µg/ml) and acridine orange (150 µg/ml) by spread plating technique. In the above concentration of chemical mutagens, growth of *Bacillus cereus* was not formed. Therefore, the concentrations of chemical mutagens were reduced till observations of the growth. Hence, the concentration of ethidium bromide and acridine orange were fixed as 25 and 100 µg/ml, respectively. The plates were then incubated at 37°C for 24 h. After incubation, the number of colonies was noted and the isolated colony from each plate was streaked and subcultured.

Chitinase assay using mutant cultures

Chitinase assay was performed for the examination of enzyme production by the selected mutant strains.

Mutagenic characterization of microbes

Bacterial culture which showed a maximum chitinolytic activitiy (*Pseudomonas alcaligenes*) was mutated by chemical means. The chemical mutation was done by chemical agents like Ethidium bromide and Acridine orange.

Chitinase assay (Tweddell et al., 1994)

Chitinolytic bacteria were inoculated individually into chitinase liquid medium ((g/l): soya bean powder, 20.0 g; starch, 4.0 g; peptone, 3.0 g; yeast extract, 2.0 g; KH_2PO_4, 0.3 g; $MgSO_4$, 0.3 g; $CaCO_3$, 1.0 g; pH, 6.5) and were incubated at 30°C for 48 to 72 h in a shaker. After incubation, the culture filtrate source was taken by centrifuging the culture fluid at 5,000 rpm for 15 min. To 1 ml of the enzyme source, 1 ml of 2% colloidal chitin was added and incubated at 50°C for 1 h. Then, 3 ml of DNS reagent was added to it and was boiled for 10 min. Finally, it was centrifuged at 3,000 rpm for 20 min and the OD of chitinase enzyme production was measured by an UV-spectrophotometer at 530 nm.

Effect of various nutrient sources, pH and temperature on chitinase production by mutant strains

The assay procedure described earlier was performed for chitinase activity by mutated strains individually using different sources of carbon (glucose, sucrose, lactose and CMC), nitrogen (NH_4Cl, NH_4SO_4, $NaNO_3$ and KNO_3), temperature (30, 35, 40 and 45°C) and pH (5.5, 6.5, 7.5 and 8.5).

Statistical analysis

The experiments were conducted following completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by Duncan's multiple range test (DMRT). Whenever necessary, the data were transformed before statistical analysis following appropriate methods.

RESULTS

Isolation and identification of gut microflora

The total viable count of bacterial colonies recorded in

Table 1. Total colonies of *Pseudomonas alcaligenes* after chemical mutation with different mutagens**.**

Chemical mutagens	Number of colonies (CFU/ml)
Control (wild strain)	115 ± 0.66^{c}
Ethidium bromide (200)	96 ± 1.20^{b}
Acridine orange (150)	40 ± 0.40^{a}

Values in parenthesis indicate concentration (µg/ml) of chemical mutagens.

the gut samples of shrimps was 115 ± 0.66. Based on the morphological, physiological and biochemical characteristics, seven bacterial strains were identified (*B. cereus, Bacillus polymyxa, Bacillus stearothermophilus, Bacillus circulans* and *Bacillus mycoides*, *Pseudomonas alcaligenes* and *Pseudomonas anguilliseptica*).

Enzymatic characterization of identified bacterial species

All the identified bacterial strains showed a positive chitinolytic activity.

Enzyme production by mutated strains

The maximum level of chitinase producing strain *P. alcaligenes* as per the results obtained in the previous experiments was mutated using ethidium bromide and acridine orange as chemical mutagens.

Chitinase production by mutated *P. alcaligenes*

Chemical mutation

When *P. alcaligenes* mutated chemically with various mutagens, the load of the colonies decreased much when compared with that of the control plate (115 ± 0.66 CFU/ml) (Table 1). Among the tested chemical mutagens, *P. alcaligenes* treated with ethidium bromide recorded a maximum of 96 ± 1.20 CFU/ml, whereas, in the acridine orange treated plate, the number of colonies observed was 40 ± 0.40 CFU/ml, respectively.

Chitinase production at different media temperature

Among the tested chemical mutagens, ethidium bromide treated *P. alcaligenes* produced maximum level of chitinase production at 35°C (118.65 ± 2.77 U/ml) (Table 2). The differences between chitinase production between the changes in chemical mutagens as well as between the changes of media temperature were found statistically significant (F = 11.365 and 26.407).

Chitinase production at different media pH

Within the chemically mutated *P. alcaligenes*, the organisms treated with acridine orange produced a maximum of 107.4 ± 0.50 U/ml at pH 7.5. The wild strain produced a maximum of 74.1 ± 1.26 U/ml at pH 7.5 (Table 3). The influence of different chemical mutagens and also the influence of different media pH on chitinase production were observed statistically more significant (F = 26.192 and 8.888; P < 0.001 to P < 0.0001).

Chitinase production at different carbon sources supplied media

Among the tested chemical mutagens, the test organisms treated with acridine orange exhibited maximum of 106.0 ± 1.89 U/ml when utilizing CMC as the carbon source. At the same time, the wild strain produced a maximum of 79.2 ± 1.14 U/ml by utilizing CMC as the sole carbon source (Table 4). The variation between chemical mutagens and the variation between tested different carbon sources on chitinase production were statistically (two-way ANOVA) significant (F = 14.872 and 11.264; P < 0.001 to P < 0.0001).

Chitinase production at different nitrogen sources supplied media

Among the tested chemical mutagens, *P. alcaligenes* treated with ethidium bromide produced a maximum of 91.2 ± 1.51 U/ml at KNO_3 supplied medium. Invariably, the enzyme production by the control strain was within the range between 63.70 ± 2.35 and 73.2 ± 2.29 U/ml at different nitrogen sources supplied media (Table 5). Chitinase production on the influence of different chemical mutagens as well as different tested nitrogen sources were statistically more significant (F = 16.088 and 9.630; P < 0.001 to P < 0.0001).

DISCUSSION

A variety of methods have been employed to modify enzymes for their industrial usage including strain improvement (Chand et al., 2004). The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. Acridine dyes are aromatic compound that intercalate within pairs of bases in the DNA molecule favouring insertions and deletions of nucleotide bases upon replication (Kapuscinski and Darzynkiewicz, 1984). In the present study, maximum (118.65 ± 2.77 U/ml) chitinase production was registered by the ethidium bromide treated mutant strain of *P. alcaligenes* grown at the incubation temperature of 35°C. Beyond this

Table 2. Chitinase production (U/ml) by the mutated strain *Pseudomonas alcaligenes* at different temperature.

Chemical mutagens	Chitinase production (U/ml) at different temperature (°C)			
	30	35	40	45
Wild strain	62.1 ± 2.43[b]	78.4 ± 2.27[a]	59.20 ± 1.54[c]	47.9 ± 0.34[d]
Chemical mutation (CM)				
Ethidium bromide mutant	66.8 ± 1.58[d]	118.65 ± 2.77[a]	93.25 ± 2.95[b]	80.65 ± 3.47[c]
Acridine orange mutant	65.55 ± 0.99[d]	103.15 ± 1.74[a]	96.2 ± 1.85[b]	74.45 ± 2.17[c]

Table 3. Chitinase production by the mutated *Pseudomonas alcaligenes* at different pH.

Chemical mutagens	Chitinase production (U/ml) at different media pH			
	5.5	6.5	7.5	8.5
Wild strain	54.8 ± 1.19[c]	68.2 ± 1.24[b]	74.1 ± 1.26[a]	53.9 ± 2.34[d]
Chemical mutation (CM)				
Ethidium bromide mutant	84.60 ± 0.77[c]	87.30 ± 0.80[b]	88.30 ± 0.82[a]	75.3 ± 1.52[d]
Acridine orange mutant	86.5 ± 0.48[c]	98.95 ± 0.51[b]	107.4 ± 0.50[a]	82.25 ± 0.95[d]

Table 4. Chitinase production (U/ml) by the mutated strain of *Pseudomonas alcaligenes* at different carbon sources media.

Chemical mutagen	Chitinase production (U/ml) at different carbon sources			
	Glucose	Sucrose	Lactose	Carboxymethyl cellulose (CMC)
Wild strain	64.1 ± 1.30[b]	59.4 ± 1.20[c]	48.9 ± 0.86[d]	79.2 ± 1.14[a]
Chemical mutation (CM)				
Ethidium bromide mutant	87.95 ± 2.69[b]	71.5 ± 1.58[d]	83.40 ± 2.47[c]	93.0 ± 3.01[a]
Acridine orange mutant	90.00 ± 7.80[b]	72.85 ± 0.99[d]	75.85 ± 1.55[c]	106.0 ± 1.89[a]

Table 5. Chitinase production (U/ml) by the mutated strain of *Pseudomonas alcaligenes* at different nitrogen sources media.

Chemical mutagens	Chitinase production (U/ml) at different nitrogen sources			
	NH_4Cl	$NaNO_2$	KNO_3	NH_4SO_4
Wild strain	68.5 ± 2.29[b]	73.2 ± 2.29[a]	66.8 ± 2.33[c]	63.70 ± 2.35[d]
Chemical mutation (CM)				
Ethidium bromide mutant	86.35 ± 1.49[c]	88.55 ± 1.49[b]	91.2 ± 1.51[a]	75.20 ± 1.53[d]
Acridine orange mutant	78.05 ± 0.93[c]	90.5 ± 0.93[a]	82.10 ± 0.95[b]	70.9 ± 0.95[d]

temperature, chitinase production reduced. It may be due to the reduction of moisture content in the production medium, subsequently the growth of the organisms was reduced and it leads to reduction of enzyme production (Haq et al., 2010). Lactose- yeast extract in the production medium acted as the best source for chitinase production (25.03% higher than wild strain) by an EMS treated mutant of *Aspergillus terreus* (Narayanan et al., 2013). In contrast, in the present study, the production medium with CMC as the carbon source supported

maximum (106.0 ± 1.89 U/ml) chitinase production by the acridine orange treated mutant of *P. alcaligenes*. It is evident from the present study that the wild strain of *P. alcaligenes* produced only 74.1 ± 1.26 U/ml of chitinase, at the same time, the strain treated with acridine orange produced 107.4 ± 0.50 U/ml enzyme. It is important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Thus, an attempt was made in the present study to mutate *P. alcaligenes* to increase chitinase production. The overall results indicated that mutant strains of *P. alcaligenes*, based on their characterization, could be useful sources of enzymes and have the potential for industrial application.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Soil chemical properties and legume-cereal rotation benefits in an Ultisol in Nsukka, Southeastern Nigeria

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This research was conducted at the Department of Soil Science, University of Nigeria Teaching and Research Farm in 2008 and 2009 growing seasons. The objective was to evaluate the effects of edible grain legumes (cowpea and soybean) and velvet-bean/maize rotations on soil chemical properties and the contribution of these chemical properties to rotation benefit conferred on the maize by velvet-bean, cowpea and soybean. The experimental design was a factorial fitted into randomized complete block design comprising of four crop rotation systems, two nitrogen levels and two residue management options as factors replicated thrice making a total of 48 plots. Each year entailed two cropping sessions, first, the four crops (cowpea, soybean, velvet-bean and maize) were grown on separate plots and after harvest; maize was grown in all the plots to test for rotation effect. The result showed that the soil chemical properties nitrogen (N) and magnesium (Mg) were significantly higher in the legume-cereal rotations than in continuous maize in both years. Other chemical properties varied in the two years and between legume/maize and continuous maize rotations. Maize yield was significantly increased by velvet-bean/maize rotation in both years. Maize grain yields were also higher in cowpea/maize and soybean/maize rotations than in continuous maize but they were not significantly different. There was also significant rotation residue interaction effect with velvet-bean/maize rotation x residue having the highest maize yield. Regression analysis showed that 37 to 51% changes in maize yield were contributed by N, Mg and potassium (K).

Key words: Legume/cereal rotation, residue management, rotation benefit, soil chemical properties, maize yield.

INTRODUCTION

Legume/cereal rotation systems have been advocated as low input management strategy to increase cereal yields on acid sandy soils of sub-Saharan West Africa that are notoriously low in phosphorus (P) and nitrogen (N). Bationo et al. (1994) stated that continuous cropping of pearl millet resulted in lower yields across all nitrogen rates than when rotated with cowpea or groundnut in different agro-ecological zones. Burkert et al. (2001) noted that legume-induced increases in cereal total dry matter as recorded in the field experiments were site and crop specific, which is relatively consistent over years, but tended to grow over time. Despite increase in cereal

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yield, incorporation of green manure crops, which fix nitrogen and produce large quantities of residual biomass, also lead to soil improvement (Chikoye et al., 2002, Honlonkou et al., 1999), but these have had low adoption rate among farmers. The low adoption rates have been related to farmers` reluctance to invest land, labour and seeds into a technology that does not provide a direct return to their investment. As a result, grain and dual- purpose legumes, such as soybean and cowpea have the advantage of giving a more rapid return to investments. However, the nitrogen contribution of grain legumes to the soil may be less than that of green manure crops and grain legumes may be less beneficial for soil fertility improvement (Schultz et al., 2001; Giller et al., 1998). Annual grain legumes perform dual function. First, they are normally grown for grain production and secondly, some producers use them as green manure crops. Generally, most recognized benefit of a legume crop to a succeeding cereal is improvement in yield. This is known as rotation benefit. This benefit results from either improvement of N and non-N soil components, both known as other rotation benefits. Nitrogen rotation benefit is the yield advantage associated with extra soil N availability to a cereal crop succeeding a legume. For example, wheat following pea accumulated approximately 50 kg/ha more N compared to wheat in a wheat/wheat rotation (Evans et al., 1991). Non-N rotation benefit in a legume/cereal rotation is that portion of the yield increase not explained by extra N accumulated by a succeeding cereal crop or that portion of the yield advantage relative to a cereal-cereal rotation that cannot be accounted for by the addition of fertilizer N (Bullock, 1992).

There is an ongoing argument as to what these 'other rotation benefits' are. Notably, proper understanding of these other rotation benefits will lead to effective management and utilization of legume-cereal rotation systems for sustainable crop improvement. These 'other rotation benefits' have been attributed to chemical and biological factors such as enhanced P nutrition (Alvey et al., 2001), weed control (Tarawali et al., 1999) and reduction of soil-borne diseases and parasitic nematodes (Vargas-Ayala et al., 2000; Bagayoko et al., 2000). Presently, a fundamental and complete understanding of the beneficial rotation benefits is lacking and remains a scientific challenge and is necessary for proper utilization of legume-cereal rotation system.

Therefore, this research evaluated the effects of edible grain legumes (cowpea and soybean) and velvet-bean rotations on soil chemical properties and effect of these chemical properties on rotation benefit conferred on the maize by the velvet-bean, cowpea and soybean. The specific objectives were to determine the effect of legume-cereal rotation on soil chemical properties, compare the beneficial effect of cowpea and soybean in relation to velvet-bean in contributing to rotation benefits in an integrated nutrient management and determine the contribution of the soil chemical properties to rotation benefit

MATERIALS AND METHODS

Site and soil description

Field experiments were conducted at the University of Nigeria, Nsukka Teaching and Research Farm, which is a derived savannah zone of Nigeria. Nsukka is located on Latitude 6^0 51′N and Longitude $7^0$24′E (Jungerius, 1964). It exhibits tropical wet (March to October) and dry (November to February) seasons with a mean annual rainfall of 1250 mm and a mean annual temperature of 26°C. The soil is degraded sandy clay and classified as Typicpaleustult belonging to Nkpologu series (Nwadialor, 1989). It is very deep, with dark reddish brown colour in the topsoil and red in subsoil. It is coarse to medium textured, granular in structure, acid in reaction and low in nutrient status. Its clay mineralogy is composed mainly of kaolinite and quartz (Akamigbo and Igwe, 1990).

Experimental design and treatments

This experiment was a rotation field trial. It was carried out in 2008 and 2009. The experimental design was factorial in randomized complete block design (RCBD), having three factors. Factor A was 4 crop rotation systems namely (velvet-bean/maize (VE/MA), cowpea/maize (CO/MA), soybean/maize (SO/MA) and maize/maize (MA/MA)), factor B was 2 nitrogen application levels (N at 0 kg ha^{-1} and N at 60 kg ha^{-1}, and factor C was 2 crop residue managements (Residue incorporated (RI) and Residue not incorporated (RN)) replicated three times making 48 plots. In each year, two cropping sessions were done, first was the cultivation of the legumes (velvet bean, cowpea and soybean) and maize as a reference crop. Second was the cultivation of maize in all the plots with and without residue incorporation and/or nitrogen application after harvesting the first crops.

The treatments were randomly applied to the plots using lettered papers. Hybrid maize seeds (*Zea mays* L.) cv Oba Super II, dual-purpose cowpea (*Vigna uniguiculata*) cv03k-374-4, early- duration soybean (*Glycine max)* cv TGX 1448 and velvet bean (*Mucuna pruriens*) were planted, three seeds per hole and thinned down to one seedling per hole after two weeks of germination. The planting spacing was 75 cm by 25 cm. The total area was 0.05 hectare. The main plot, subplot and sub-sub plots were 5 x 6 m, 2.5 x 6 m and 2.5 x 3 m, respectively. Weeding was done twice during the period. In 2008, the first and second planting took place on 23 June and September 5, respectively. The date for maize harvest was on 21 November whereas in 2009 the first and second cultivation took place on 8th June and 9th September, respectively. The plants were harvested at maturity in late November.

Data collection

At the onset of the experiment in 2008, representative soil samples were collected from 0 to 15 cm from the topsoil of the area and mixed together to obtain a composite sample. In addition, soil samples were collected at the end of each harvest. The entire soil samples were air-dried, sieved with 2 mm sieve and subjected to chemical soil analyses. Soil pH was measured potentiometrically in 1:2.5 soil to water ratio with the glass electrode pH meter (McLean, 1982); organic carbon (OC) by the Walkley and Black wet dichromate oxidation method (Nelson and Sommers, 1982), organic matter (OM) was calculated by multiplying organic carbon figure by the conventional "Van Berminelen factor" of 1.724; exchangeable

Table 1. The properties of soil of the location.

Parameter	UNN
Clay (%)	38.6
Silt (%)	17.6
Sand (%)	43.9
Textural class	Sandy clay
BD	1.10
pH	5.2
N (%)	0.11
OC (%)	0.86
OM (%)	1.48
P (mg kg^{-1})	6.48
Ca ($cmolkg^{-1}$)	0.8
Mg ($cmolkg^{-1}$)	0.6
K ($cmolkg^{-1}$)	0.09
Na ($cmolkg^{-1}$)	0.07
EA ($cmolkg^{-1}$)	1.4
ECEC ($cmolkg^{-1}$)	2.96
CEC ($cmolkg^{-1}$)	5.3
BS (%)	52.7
C/N	9.8

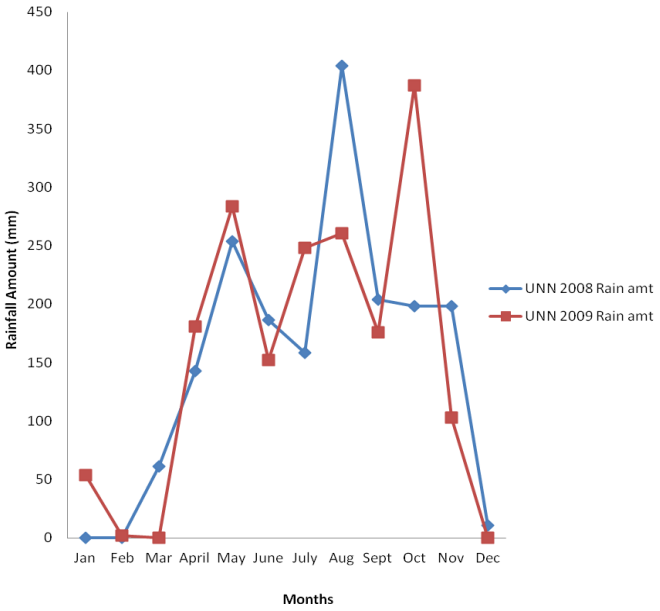

Figure 1. Rainfall amount for 2008 and 2009 at Moniya-Ibadan and UNN.

bases by extraction with neutral 1 N NH_4OAc. Potassium (K) in the extract was determined with flame photometer (Kundsen et al., 1982), Ca and Mg by atomic absorption spectrophotometer (L). Exchangeable acidity was determined by the KCl displacement method described by Page et al. (1982). Effective cation exchange capacity (ECEC) was obtained from the sum of the exchangeable bases and exchangeable acidity. Available P was extracted by Bray II method; the P concentration in the extract determined colorimetrically using the spectronic 70 spectrophotometer method (Page et al., 1982). Total N in soils was determined by the Micro-Kjeldahl digestion procedure (Bremner and Mulvaney, 1982).

Plant residue laboratory analysis

The residue for maize and velvet-bean were analyzed for total N, P, K, Ca, Mg and organic carbon. Total N was by the method of Bremner and Mulvaney (1982). For other analyses, 0.5 g of the residue was extracted with 20 ml concentrated nitric acid and allowed to stay overnight. It was then digested until blackish organic matter disappears. Then, 20 ml of H_2O_2 was added as digestion continued until white fumes appeared indicating complete digestion. Water was then added and filtered to 100 ml volume. The filtrate was used for determination of total P, K, Ca and Mg according to methods for soil sample determination as enumerated above. Maize crops were harvested maturity dried before shelling to obtain dry matter and grain yield. All plant samplings were done by cutting the shoot at soil level.

RESULTS AND DISCUSSION

Physicochemical properties of the soil at the beginning of the experiment are shown on Table 1. The pH of UNN soil is strongly acid (5.1) (USDA – SCS, 1994). The exchangeable Ca, Mg, K, Na, and cation exchange capacity of UNN soil were very low. Total N of 0.07% was very low (Landon, 1991) and available P was also low (6.48 mg/kg). The low nutrient content of UNN soils may have resulted from very high rainfall that leaches these nutrients with high temperatures, almost all the periods of the year. For instance, there was 404.15 mm of rainfall in 18 days in 2008 and in October 2009, there was 387.1 mm of rainfall in 17 days which were rather too high (Figure 1).

Chemical properties of the residues

Table 2 shows the chemical properties of the crop residues. Total N, P, Ca and Mg were higher in velvet-bean residue but total K and organic C were higher in maize residue. Therefore, velvet-bean residue contains more nutrients needed by crops for their growth and development. In addition, velvet-bean residue had lower C/N and C/P ratios, depicting easier degradation and earlier release of nutrients.

Soil chemical properties as affected by legume-cereal rotation, residue incorporation and nitrogen application

In 2008, Legume-cereal rotations significantly ($p < 0.05$) affected pH, OC, N, Ca, Mg, EA, ECEC, pH, OC and EA (Table 3) and pH, P, N, Ca, Mg, EA, pH, and EA in 2009. These soil chemical properties: N, Ca, Mg and ECEC

Table 2. Characteristics of organic wastes used in this study.

Parameter measured	Mucuna residue	Maize residue
Organic C (%)	5.8	6.7
Total N (%)	2.7	2.1
Total P (%)	2.6	2.2
Total K (%)	0.8	1.2
Total Ca (%)	0.4	0.2
Total Mg (%)	2.5	1.5
C/N	2.15	3.19
C/P	2.23	3.05

Table 3. Soil chemical Properties as affected by cropping system, residue application and N rates at the end of the experiment in UNN.

Parameter	pH	OC (%)	N (%)	P (mg/kg)	Ca	Mg	K (cmol/kg)	EA	ECEC
Cropping systems				**2008**					
Velvetbean/Maize	4.51	0.98	0.107	13.87	0.83	0.59	0.24	1.08	2.86
Cowpea/maize	4.51	1.19	0.098	15.32	0.73	0.54	0.18	0.86	2.42
Soybean/Maize	4.45	0.99	0.097	13.46	0.70	0.50	0.22	1.00	2.52
Maize/maize	4.7	1.01	0.070	14.32	0.63	0.47	0.21	0.92	2.33
LSD (0.05)	0.08**	0.11**	0.01**	ns	0.08**	0.05**	ns	0.14*	0.18**
N application									
Zero N	4.52	1.02	0.09	14.07	0.70	0.51	0.24	0.90	2.45
60 Kg/ha N	4.57	1.07	0.1	14.42	0.74	0.54	0.19	1.04	2.61
LSD (0.05)	ns	ns	ns	ns	ns	ns	ns	0.1*	0.13**
Residue addition									
Zero Residue	4.55	1.04	0.087	13.33	0.70	0.49	0.22	1.02	2.54
Residue addition	4.54	1.05	0.098	15.17	0.74	0.56	0.21	0.91	2.52
LSD (0.05)	ns	ns	0.007**	ns	ns	0.03**	ns	0.1*	ns
Cropping systems				**2009**					
Velvetbean/Maize	4.59	0.95	0.070	13.01	0.99	0.22	0.16	1.28	2.75
Cowpea/maize	4.45	0.99	0.063	13.17	1.21	0.26	0.14	0.93	2.67
Soybean/Maize	4.61	0.94	0.063	15.64	1.15	0.24	0.14	1.16	2.80
Maize/maize	4.41	0.89	0.045	10.23	1.00	0.19	0.14	1.21	2.67
LSD (0.05)	0.16*	ns	0.007**	3.42*	0.17*	0.03**	ns	0.1**	ns
N application									
Zero N	4.50	0.93	0.058	13.09	1.08	0.23	0.13	1.07	2.65
60Kg/ha N	4.53	0.95	0.062	12.93	1.09	0.23	0.15	1.22	2.81
LSD (0.05)	ns	ns	ns	ns	ns	ns	ns	0.07**	0.12**
Residue addition									
Zero Residue	4.56	0.93	0.056	13.19	1.10	0.22	0.14	1.20	2.78
Residue addition	4.46	0.94	0.065	12.83	1.08	0.24	0.15	1.09	2.68
LSD (0.05)	ns	ns	0.005**	ns	ns	ns	ns	0.07**	ns

ns = not significant, * = significant at 5%, ** = significant at 1%

Table 4. Interaction effect between cropping system and residue application at UNN.

Cropping system x Residue	pH	OC (%)	N (%)	P (mg/kg)	Ca	Mg	K (cmol/kg)	EA	ECEC
2008									
Va/Ma X R	4.6	1.02	0.11	13.89	0.80	0.61	0.22	0.90	2.63
Cp/Ma X R	4.5	1.18	0.10	16.32	0.77	0.57	0.20	0.90	2.55
Sb/Ma X R	4.5	0.96	0.11	14.72	0.69	0.53	0.20	1.00	2.52
Ma/Ma X R	4.7	1.01	0.08	15.74	0.70	0.51	0.22	0.86	2.39
Va/Ma X NR	4.6	0.94	0.10	13.89	0.85	0.57	0.26	1.27	3.09
Cp/Ma X NR	4.5	1.19	0.10	14.32	0.68	0.51	0.17	0.82	2.29
Sb/Ma X NR	4.4	1.01	0.09	12.19	0.69	0.46	0.24	1.00	2.52
Ma/Ma X NR	4.8	1.01	0.06	12.90	0.56	0.43	0.20	0.97	2.28
Lsd (0.05)	0.1*	ns	ns	ns	0.12**	ns	ns	0.19*	0.25**
2009									
Va/Ma X R	4.4	0.98	0.08	13.6	1.1	0.3	0.18	1.0	2.6
Cp/Ma X R	4.3	0.95	0.07	17.6	1.1	0.3	0.13	1.0	2.7
Sb/Ma X R	4.6	0.93	0.07	14.6	1.0	0.2	0.15	1.2	2.6
Ma/Ma X R	4.6	0.91	0.05	11.2	1.1	0.2	0.13	1.2	2.7
Va/Ma X NR	4.4	0.92	0.07	14.8	0.9	0.2	0.14	1.5	2.9
Cp/Ma X NR	4.6	0.98	0.06	14.4	1.3	0.3	0.15	1.0	2.7
Sb/Ma X NR	4.6	0.95	0.06	16.7	1.3	0.3	0.13	1.2	3.0
Ma/Ma X NR	4.6	0.90	0.04	9.3	0.9	0.2	0.14	1.3	2.6
Lsd (0.05)	ns	ns	0.01**	ns	0.24**	0.05**	0.03**	0.21**	0.29*

* = Significant at 5%, ** highly significant at 1%, ns- not significant. Va-velvet-bean, CP- cowpea, SB- soybean MA- maize, R- Residue addition NR- no residue addition

were higher in the legume-cereal rotations than in continuous maize in 2008 with velvet-bean/maize rotation having significantly higher values. Velvet-bean/maize rotation had 0.107% N, 0.83 cmol kg^{-1} Ca, 0.59 $cmolkg^{-1}$ Mg and 2.86 $cmolkg^{-1}$ ECEC, followed by cowpea-maize (0.098% N, 0.73 $cmolkg^{-1}$ Ca, 0.54 $cmolkg^{-1}$ Mg and 2.42 $cmolkg^{-1}$), soybean-maize rotations (0.097% N, 0.70 $cmolkg^{-1}$Ca, 0.50 $cmolkg^{-1}$ Mg and 2.52 $cmolkg^{-1}$ ECEC) with maize having the least values (0.070% N, 0.63 $cmolkg^{-1}$ Ca, 0.47 cmol kg^{-1} Mg and 2.33 cmol kg^{-1} ECEC). Continuous maize had the highest pH of 4.7 and soybean-maize rotation, the least value of 4.45. Organic C and EA varied among the legume-cereal rotations and continuous maize. Organic C was highest in cowpea-maize rotation (1.19%) and the least was velvet-bean-maize rotation (0.98%), soybean-maize had 0.99% OC and continuous maize 1.01%. Similar trend was observed in EA with the least value obtained from cowpea-maize rotation (0.86 $cmolkg^{-1}$) and the highest value in velvet-bean-maize rotation (1.08 $cmolkg^{-1}$). Similar result was obtained in 2009 but pH, avail P and Mg were higher in legume/maize rotations but Ca and ECEC varied.

Nitrogen application affected significantly EA and ECEC, with N application having higher values (1.04, 1.22 and 2.61, 2.81 $cmolkg^{-1}$) than no application (0.90, 1.07 and 2.45, 2.65 $cmolkg^{-1}$) in both years. Residue addition significantly affected N, Mg and EA, with residue addition having higher values than no addition except for EA, which was higher in no addition of residue. This was also the case in 2009. The interaction between cropping system and residue incorporation (Table 4) shows that the Legume x residue highly increased soil N, Ca and Mg than maize residue. In addition, for N specifically, legume-cereal rotations gave higher soil N than continuous maize whether there was addition of residue or not. The legume-cereal X legume residue interaction improved most of these parameters over continuous maize X maize residue interaction.

The lower pH in the legume/cereal rotation in soils in 2008 agrees with the findings of Helyar and Porter (1989), which states that the presence of legumes in agricultural system influences soil acidity through the N and C cycles. Legumes increase soil organic N through N-fixation, and subsequent oxidation of organic N followed by NO_3 leaching is the main acidifying process (Helyar, 1976). Secondly, the excretion of H^+ from legume roots, due to the uptake of more cations than anions (Haynes, 1983), is another reason for accelerated acidification associated with legume growth. At Tarlee site, the results show that the wheat-lupin rotation gave the highest acidification rate. Tang et al. (1998) have shown that the total acid excretion by the roots of some

Table 5. Dry matter (t/ha) and grain yield (t/ha) as affected by cropping system, residue application and N rates.

Treatment	Dry matter (t/ha)		Grain yield (t/ha)	
	2008	2009	2008	2009
Cropping systems				
Velvet-bean/Maize	32.17	31.34	1.85	2.04
Cowpea/maize	10.86	9.49	0.55	0.65
Soybean/Maize	9.17	10.82	0.62	0.57
Maize/maize	9.20	8.20	0.52	0.52
LSD (0.05)	2.59**	2.90**	0.16**	0.06**
Nitrogen application (N)				
Zero N	10.46	12.63	0.62	0.81
60Kg/ha N	20.23	17.30	1.15	1.08
LSD (0.05)	1.83**	2.05**	0.12**	0.04**
Residue				
Zero Residue	10.59	11.42	0.51	0.67
Residue addition	20.11	18.50	1.26	1.22
LSD (0.05)	1.83**	2.05**	0.12**	0.04**

* Significant at 5%, highly significant at 1%, ns- not significant

Table 6. Interaction effect of cropping system and residue application.

Treatment interaction	Dry matter (t/ha)		Grain yield (t/ha)	
	2008	2009	2008	2009
Velvet-bean/Maize *Residue	40.28	37.36	2.61	2.79
Velvet-bean/Maize*Zero Res	24.06	25.31	1.09	1.29
Cowpea/maize*Residue	14.98	12.44	0.79	0.86
Cowpea/maize*Zero Residue	6.73	6.54	0.32	0.44
Soybean/Maize*Residue	11.50	14.64	0.76	0.66
Soybean/Maize*ZeroRes	6.84	7.00	0.47	0.47
Maize/Maize*Res	13.66	9.57	0.90	0.55
Maize/Maize*ZRes	4.73	6.84	0.14	0.49
LSD(0.05)	3.37**	3.60*	0.19**	0.16**

* Significant at 5% ** highly significant at 1%, ns- not significant.

pasture legumes correlated with the total shoot content of excess cations, and this was associated with a decrease in soil exchangeable base cations. In addition, higher nutrient in legume-cereal rotation over continuous maize agrees with the work of Alvey et al. (2001); Muhr et al. (1999) that mineral nutrition of the soil is increased by the legume because of higher solubilization of the occluded nutrients.

Effect of the treatments on maize yield

Table 5 shows the effects of cropping systems, residue additions and N applications on maize yield. Comparing the cropping systems, velvet-bean/maize rotation produced significantly higher maize grain yields (1.85 tha^{-1} in 2008, 2.04 tha^{-1} in 2009), which was statistically different from all other rotations. Velvet-bean/maize rotation is notable in significant improvement in subsequent maize yield (Buerkert et al., 2001). In 2008, cowpea/maize and soybean/maize rotations were significantly the same with continuous maize yield but in 2009, cowpea/maize was significantly higher than continuous maize in increasing maize yield but soybean/maize rotation did not increase maize yield significantly. The generally non-significant maize yield produced in soybean and cowpea/maize rotations were because of the poor growth due to the heavy rainfall during the first cultivation in which these legumes were grown. However, the yields were still generally higher than maize/maize rotations. Velvet-bean grew very well even under heavy rainfall, so produced significant higher maize yield in rotation.

Interactions between cropping systems and residue addition significantly affected the subsequent maize yield (Table 6). Velvet-bean residue was superior to all other

Table 7. Maize yield improvement of legume/maize over maize/maize (Rotation benefit).

Treatment	2008 (%)	2009 (%)
Velvet-bean/maize	255	293
Cowpea/maize	5.7	25
Soybean/maize	19	9.6
Velvet-bean/Maize *Res	192	350
Cowpea/maize*Res	NA	115
Soybean/Maize*Residue	NA	17

Table 8. Regression equation and R^2 of significant soil factors of legume/cereal rotations.

Year	Yield parameter	R^2	Regression equation
2008	Dry matter	0.51	Y= -46.75 + 211.41N + 81.02 Mg
	Grain yield	0.44	Y= -2.82 + 15.21N + 4.37 Mg
2009	Dry matter	0.37	-23.86 + 349.41 N + 124 K
	Grain yield	0.41	-1.97 + 23.06 N + 10.65 K

N = nitrogen, Mg = magnesium, K=potassium.

residue in increasing maize yield. Table 7 shows the overall contribution of the cropping systems and their residue on rotation benefit. Velvet-bean/maize rotation had over 100% increases in maize yield in relation to continuous maize rotation. Cowpea/maize rotation had about zero to 33% increase and soybean/maize rotation had about zero to 43%.

Percentage contribution of non-N rotation benefit vis-à-vis soil chemical properties to maize yield increase

Table 8 shows the Regression coefficient (R^2) and regression equation of the second maize yield and soil chemical properties in legume-cereal rotations. In 2008, 51, 44% changes in dry matter and grain yield were contributed by N (8%) and Mg (43%) and N (34%) and Mg (10%), respectively, while in 2009, 37 and 41% changes in dry matter and grain yield were contributed by N (6%) and K (31%) and N (5%) and K (36%), respectively.

Principal component analysis of the yield variants (soil chemical properties)

Looking at the yield varieties holistically in a multivariate analysis using PCA, a comparison between soil chemical properties after the legume/cereal rotation was done. In 2008, 69% variation in the database was dealt with in third PCs at the end of the legume/cereal rotation cropping. More so, in 2009, 60% variation was dealt with after the legume/cereal rotations. Notably, in PCA, the factors with higher weight (0.30 and above) controls more of the variation. Subsequently, in 2008 at the end of the legume/cereal rotations, N, Ca, Mg, EA, ECEC (first PC), pH, OC, N, K, EA (in second PC) and pH, P, Mg, Na (in the third PC) controlled the variations. In 2009 (Table 9), similar observations were made.

Conclusions

Based on the findings of this research, there was improvement of the soil chemical properties by legume/cereal rotation cropping systems. The soil properties significantly ($p < 5\%$) affected by the legume/cereal rotations were pH, OC, N, Ca, Mg, EA and ECEC in 2008 and pH, N, P, Ca, Mg and EA in 2009. Most of these properties were higher in legume/cereal plots than in continuous maize plots with velvet-bean/maize plots having higher values. Maize yield was also increased significantly by legume/cereal rotation over cereal/cereal rotation, thus the rotation benefit. Legume/cereal rotation conferred significant rotation benefit on the cereal component over cereal/cereal rotation. The extent of the rotation benefit depended on the type of leguminous crop. Overall rotation benefit ranged from 0% to over 200%. This rotation benefit resulted from N, P, Ca, Mg and K. The non-N factors were higher than the N rotation benefit except when residue was added. Edible legumes increased maize yield in rotation over continuous maize especially in the second year though velvet-bean was superior over them in increasing maize yield. Velvet-bean/maize rotation is therefore recommended though soybean and cowpea

Table 9. Principal component analysis of soil factors of legume/cereal rotation.

Soil properties	2008			2009		
	Prin1	Prin2	Prin3	Prin1	Prin2	Prin3
pH	-0.16	-0.31	0.56	0.21	0.32	-0.52
OC	-0.12	0.45	0.15	0.36	-0.08	0.24
N	0.39	0.36	-0.13	0.24	-0.08	0.60
P	-0.08	0.27	0.58	0.27	-0.14	-0.00
Ca	0.44	0.18	-0.17	0.49	-0.15	-0.34
Mg	0.34	0.38	0.31	0.50	-0.21	0.07
K	0.18	-0.39	-0.19	0.16	0.14	0.36
Na	0.26	-0.20	0.30	-0.14	-0.33	0.07
EA	0.34	-0.31	0.23	-0.10	0.67	0.25
ECEC	0.53	-0.19	0.11	0.41	0.47	-0.01
Eigval	3.07	2.09	1.19	2.88	1.72	1.56
Prop	0.31	0.21	0.12	0.28	0.17	0.15
Cum	0.31	0.52	0.63	0.28	0.45	0.60

can also be planted in rotation with maize instead of continuous cropping of maize but rotation benefit is not as much as in the case of velvet-bean/maize rotation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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In vitro spore germination and gametophytic growth development of a critically endangered fern *Pteris tripartita* Sw.

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The effects of sucrose, pH and plant growth hormones on spore germination percentage and gametophyte growths of *Pteris tripartita* were studied. Various morphological structures of gametophytes were observed namely, filamentous, spatulate and heart stages in the MS culture medium with hormones. After 15 days, the spores of *P. tripartita* were sprouted in MS basal medium fortified with pH, sucrose and hormones. Maximum spore germination rates (84%) were observed in 70 g/L of sucrose and 79.33% in pH 5.7. On the other hand, the maximum gametophyte sizes were observed both in 40 and 50 g/l of sucrose on half strength MS medium. The maximum growth of gametophyte lengths (484.39 and 507.72 µm) and widths (846.58 and 1270.98 µm) were observed in both pH 5.7 and 6.7. Among three different hormones, the utmost number or percentage of spores were sprouted in GA_3. However, the *in vitro* cultures of spore having the capability to increase the spore germinated due to addition of adequate nutrition in the culture medium and also reduce the contamination as well as environmental factors.

Key words: *Pteris tripartita*, spore germination, sucrose, hormone, MS medium.

INTRODUCTION

Pteridophytes have alternation of generations, possessing distinct free-living diploid sporophyte and haploid gametophyte generations. The sporophyte forms are mainly used for various purposes such as ornamental, ethnobotanical, medicinal properties and mainly to maintain micro-ecological habitats (Martin et al., 2006). The free-living fern sporophytes are typically similar to vascular plants with laminate photosynthetic structures; on the other hand, fern gametophytes are very small and lack tissue organization only with a protonemal structure formed after germination of the spore (Fernandez et al., 2012). From conservation point of view, ferns are becoming endangered as the spores have difficulty to germinate under natural condition due to environmental factors. Fern spore germination and gametophyte development characteristics are much more helpful in taxonomic and phyletic studies (Raine et al., 1996; Chiou and Farrar, 1997; Chiou et al., 1998; Chandra et al., 2003). In spore culture, type of spore germination, development of the prothallial plate and the

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meristematic regions, form of the mature and old thallus, type, position and time of appearance of hairs when present and form of the sex organs (especially the antheridium) are proven valuables to the taxonomists. The earliest fern spore germination pattern was carried out in *Schizaea pusilla* (Britton and Taylor, 1901) and *S. bifida* (Thomas, 1902). Perez-Garcia and Mendoza-Ruiz (2004) indicated that gametophytes may be useful for taxonomic and phyletic studies at the family and generic levels as well as among species within the same genus. The genus *Pteris* L. (Pteridaceae) is estimated to comprise about 250 species and found in the tropics (Smith et al., 2006). Ferns from threatened ecosystems require special attention for their rescue and recovery. In the case of chlorophyllous fern spores, germination is faster than brown spores of other species. Mature brown spores have less viability than green spores but dry storage at 4 or -20°C is effective in many cases and cryopreservation is the only effective storage method for culture studies (Pence, 2000). The present investigation was focused on the effects of sucrose, pH and plant growth hormones on spore germination and their gametophyte growths of a critically endangered fern, *P. tripartita.*

MATERIALS AND METHODS

Collection and storage of spores

The matured sporophytes of *P. tripartita* were collected from matured fronds in Alagar hills, Madurai district and confirmed with the help of reference standards at Centre for Biodiversity and Biotechnology, St. Xavier's College, Palayamkottai, Tamil Nadu, India. The voucher specimen (XCH 25403) was numbered and deposited at St. Xavier's Herbarium. The fertile fronds were dried in shade condition for two days and spores were collected. The spores lose their viability if stored at room temperature. Therefore, collected spores were preserved at low temperature (4°C) for further studies. The spores were sown in culture medium within one month of their collection.

Spore germination

Before inoculation process, 5 mg of spores scooped from the storage bag and immersed in water for 2 h. Spores were sterilized with commercial bleach solution (NaClO, 0.5% v/v) with double distilled water for 10 min. All the spores were rinsed at least three times with sterile double distilled water and then they were centrifuged at 3,000 rpm for 3 min. After that, spores were collected in a sterile condition and cultured in 25 ml culture tubes (Borosil, India) containing 10 ml of Murashige and Skoog (1962) basal medium augmented with 3% (w/v) sucrose and 0.7% (w/v) agar and pH was adjusted to 5.7 with 0.1N NaOH and 0.1N HCl. Various strengths of sucrose concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 g/l), pH ranges (3.7, 4.7, 5.7, 6.7, 7.7) and plant growth hormones such as 6-benzylaminopurine (BAP), kinetin (KIN) and gibberellic acid (GA_3) (1, 2, 3, 4, 5 mg/l) were tested for their efficacy. All the cultures were maintained at 25°C under cool-white fluorescent light (40 µmol m^{-2} s^{-1}) with 16 h artificial photoperiod (Philips, India). After two weeks, hundred spores were scored per treatment to study the spore germination rate and 10 gametophytes were randomly selected and observed for the measurement of gametophytes growth.

Microscopical studies

The effects of sucrose, pH and hormones in spore germination after two weeks and gametophyte growth rates (length, width of gametophytes) were assessed after 45 days. Spore germination was scored after 14 days of inoculation and photographed in light microscope (Deep vision, India) equipped with an ocular micrometer. The morphological studies of the gametophytes were measured with the help of stereomicroscope (Nikon SMZ800, Japan) after 45 days of inoculation and photographed using stereomicroscope (Nikon Eclipse E200, Japan). The micromorphological characters such as meristematic zone, rhizoid formation, perine, obconic cell and shape of protonemal cell were also observed.

Statistical analysis

Results were represented as Mean±Standard error. Hundred spores were scored per plate for spore germination percentage and ten gametophytes were randomly selected and measured using microscopes. Spore germination percentage and its gametophyte growth values represent the average of three replicates. The analysis was carried out using SPSS 17.0 software (Chicago, USA) with one way ANOVA test with Duncan's multiple range test (DMRT) along with $p < 0.05$ as the limit of significance.

RESULTS AND DISCUSSION

Spore germination using tissue culture methods permits a spore population free from contamination by spores of other species as well as infection by bacteria, fungi, algae and mosses which usually constitute a major problem when growing under normal field conditions (Deberg, 1994). Thus, fern spore culture has been studied on *in vitro* condition to evaluate the effects of sucrose, pH and plant growth regulators on both spore sprouting rate and gametophytes growth. Moreover, these physical and chemical factors affect the processes involved in growth and development of prothallium (Fernandez et al., 1997a, b, 1999). Different morphological structures like filamentous, spatulate and heart shaped in gametophyte development of ferns were observed after 30-35 days (Delfin, 1998). However, gametophytic generation is essential in the fern life cycle and very little is known about its ecology and physiology (Greer and McCarthy, 1999; Johnson et al., 2000; Watkins et al., 2007a, b). In the present study, germination of each spore produced one gametophyte that evolved from one dimensional filamentous stage to two dimensional heart shaped stage. Germinated spores of *P. tripartita* also raised filamentous prothalli and later developed into heart shaped gametophytes.

Effect of sucrose on spore germination

According to Dyer (1979), sucrose is generally used as

Table 1. Effects of sucrose and pH on spore germination of *P. tripartita* Sw. in half strength MS medium after two weeks.

Treatment	Spore germinations (%)	Rhizoid formations (%)
Sucrose (g/L)		
0	36.33±0.88^{e}	-
10	45.00±1.15^{d}	-
20	50.00±1.52^{d}	-
30	60.66±3.38^{c}	-
40	70.33±1.45^{b}	1.33±0.33^{b}
50	68.33±2.02^{b}	4.33±0.88^{a}
60	59.66±1.45^{c}	1.66±0.33^{b}
70	84.00±1.73^{a}	2.33±0.88^{ab}
80	49.66±2.02^{d}	-
90	55.66±0.88^{c}	-
100	38.33±1.45^{e}	-
pH		
3.7	32.33±2.60^{d}	-
4.7	59.66±2.40^{c}	-
5.7	79.33±1.76^{a}	6.33±0.88^{a}
6.7	68.33±2.02^{b}	3.66±0.66^{b}
7.7	67.66±1.20^{b}	2.66±0.33^{b}

Data are shown as Mean±SE of triplicate values. Each replication consists of hundred spores. Means followed by the same letter within columns are not significantly different at P≤ 0.05 by Duncan's Multiple Range Test (DMRT).

carbon source to promote gametophyte growth of leptosporangiate ferns. Varied concentration of sucrose (0-100 g/L) was added with to ½ strength MS basal medium to study the effects of sucrose on germination of spore, since sucrose is an indispensable supplement of all plant tissue culture media to augment the carbon supply and the photosynthetic ability of the cultured tissue which is also influenced by low irradiance and gaseous exchange (Kozai, 1991; Capellades et al., 1991). Sucrose concentrations in the culture medium play a vital role in ferns tissue culture and are particularly involved in spore germination. Spores of *P. tripartita* were sprouted after 15 days of inoculation in MS basal culture medium. Maximum spore germination rate (84%) was observed with 2.33% of rhizoid formation in 70 g/L of sucrose (Table 1). Sheffield et al. (2001) and Wu et al. (2010) proved significant increase in spore germination and gametophyte growth of fern species by addition of sucrose in the medium. Furthermore, stimulation of *Platycerium bifurcatum* (Camloh, 1993) and *Osmunda regalis* (Fernandez et al., 1997b) gametophytes were increased by the addition of sucrose. Moderate spore germination rates (70.33 and 68.33%) were achieved in both 40 and 50 g/L of sucrose concentrations. Lowest spore germination rates (36.33 and 38.33%) with absence of rhizhoid formation have been noticed in both 10 and 100 g/L of sucrose. The present results directly overlap with previous outcome that the spore germination was lower with high amount of sucrose (Renner and Randi, 2004).

Effect of sucrose on gametophyte growth

The influence of sucrose on gametophyte growth was studied by the addition of sucrose (10-50 g/L) with ½ MS strength medium. Lowest gametophyte growths were observed in both concentrations 10 and 20 g/L of sucrose. At 30 g/L of sucrose, the gametophyte length was 445.51 µm with 1392.08 µm of width which showed moderate rhizoid length (309.96 µm) with 6.62 mean numbers of rhizoids. Furthermore, maximum growth rate of gametophyte lengths of 691.03 and 722.14 µm along with widths 1845.36 and 1950.91 µm were also observed in 40 and 50 g/L of sucrose on half strength MS medium which showed significant rhizoid lengths (376.62 and 436.62 µm) and rhizoid mean numbers (7.10 and 8.23). Among the five concentrations of sucrose (10-50 g/L), minimal growths of gametophytes were identified in both 10 and 20 g/L of sucrose. In 10 g/L of sucrose, 291.07 µm

Table 2. Effects of sucrose and pH on spore derived gametophytic growths of *Pteris tripartita* Sw. after 45 days of culture.

Treatment	Gametophyte length (µm)	Gametophyte width (µm)	Rhizoidal number	Rhizoidal length (µm)
Sucrose (g/L)				
10	291.07 ± 4.00^{c}	445.50 ± 7.77^{c}	-	-
20	321.07 ± 5.87^{c}	482.17 ± 10.60^{c}	2.63 ± 0.17^{c}	112.21 ± 6.75^{c}
30	445.51 ± 16.36^{b}	1392.08 ± 195.33^{b}	6.26 ± 0.66^{b}	309.96 ± 40.54^{b}
40	691.03 ± 12.81^{a}	1845.36 ± 15.43^{a}	7.10 ± 0.36^{b}	376.62 ± 16.44^{ab}
50	722.14 ± 7.28^{a}	1950.91 ± 25.83^{a}	8.23 ± 0.17^{a}	436.62 ± 19.52^{a}
pH				
3.7	158.87 ± 2.22^{c}	433.28 ± 6.93^{c}	-	-
4.7	426.62 ± 10.18^{b}	811.02 ± 18.98^{b}	-	-
5.7	484.39 ± 8.67^{a}	846.58 ± 23.33^{b}	32.13 ± 0.46^{a}	443.28 ± 15.02^{a}
6.7	507.72 ± 14.18^{a}	1270.98 ± 39.73^{a}	27.70 ± 0.86^{b}	451.06 ± 7.28^{a}
7.7	407.73 ± 8.01^{b}	853.24 ± 15.74^{b}	28.43 ± 0.98^{b}	394.39 ± 14.44^{b}

Data are shown as Mean±SE of three replications. Each replication consists of 10 gametophytes. Means followed by the same letter within columns are not significantly different at P≤ 0.05 by Duncan's multiple range test (DMRT).

of gametophyte length and 445.50 µm of width with the absence of rhizoids were observed. The gametophyte length (321.07 µm) and width (482.17 µm) with lowest growth of rhizoid length (112.21 µm) and rhizhoid mean numbers (2.63) were noticed in 20 g/L of sucrose (Table 2). Fernandez et al. (1999) observed the effect of sucrose on gametophyte development along with the presence of mineral salts in the culture medium. Moreover, the addition of sucrose to the medium not only increases gametophytic morphological growth and shoot number but also, increase embryo induction that ultimately shortening the period from gametophyte to sporophytes of both *Nephrolepis biserrata* and *Aleuritopteris argentea* (Ambrosio and De Melo, 2004; Huang et al., 2009). The results revealed that the length, width, rhizoid length and its numbers of *P. tripartita* could be increased by the addition of sucrose in ½ strength MS culture medium condition.

Effect of pH on spore germination

The influence of pH over the multiplication efficiency, establishment and quality of the propagated sporophytes of ferns as well as ornamental plants were well documented (Handreck, 1992; Koedam et al., 1992; Pevalek-Kozlina, 1996; Symonds et al., 2001; Ambrosio and De Melo, 2004). Maximum spore sprouting rate (79.33%) was achieved along with 6.33% of rhizoid formation in pH 5.7 among five different pH ranges (3.7-7.7). Subsequently, minimum spore germination rates (68.33 and 67.66%) were observed with 3.66 and 2.66% of rhizoid formations at pH of 6.7 and 7.7, respectively (Table 1). In earlier studies, spores of *Drynaria fortunei* showed highest germination frequency occurred at the pH toward alkalinity (pH 7.7) (Raghavan, 1989; Chang et al., 2007). In both pH ranges 3.7 and 4.7, the spore germination rates were 32.33 and 59.66% with the absence of rhizoid formations, respectively. The present results concluded that increasing pH range in MS culture medium plays significant role on spore germination and its gametophyte growth.

Effect of pH on gametophyte growth

The influence of pH in culture medium significantly affects the gametophyte growths. Due to acidic conditions of MS culture medium, there were no rhizoidal formation in lowest pH ranges (3.7 and 4.7), but showed the lowest gametophyte lengths (158.87 and 426.62 µm) and widths (433.28 and 811.02 µm), respectively. The minimum growth of gametophyte was observed in pH 7.7 in which, 407.73 µm of length and 853.24 µm of width with 394.39 µm of rhizoidal length along with 28.43 mean numbers of rhizoid were also noticed. Moreover, maximum growth of 484.39 and 507.72 µm of gametophyte lengths and 846.58 and 1270.98 µm of widths of gametophytes were observed in both pH ranges of 5.7 and 6.7, correspondingly (Table 2). Earlier report also proved that pH 6.7 provided better culture condition for the gametophyte development of *Sphaeropteris lepifera* (Ma et al., 2010). In the pH ranges 5.7 and 6.7, mean numbers of rhizoid (32.13 and 27.70) were also noticed (Figure 1).

Effect of hormones on spore germination

Generally, spore germination capability increased while MS media was supplemented with hormones, sugar and casein hydrosylate (Renner and Randi, 2004; Mazumder

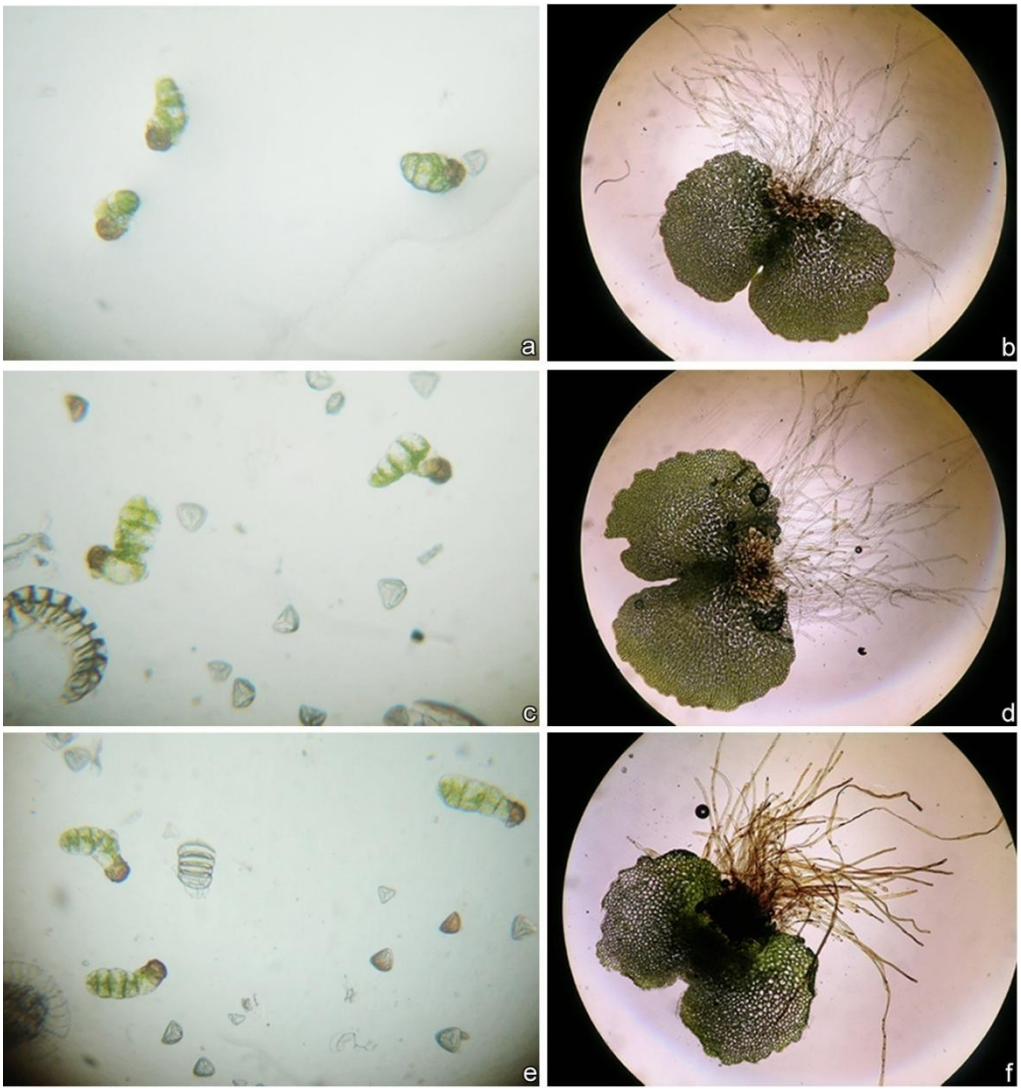

Figure 1. Effect of pH and sucrose on spore sprouting and gametophyte growth of *Pteris tripartita* Sw. (a) Germinated spores cultured in pH 5.7, (b) Heart shaped gametophyte in pH 5.7 after 45 days, (c) Germinated spores in pH 7.7 culture medium, (d) Heart shaped gametophyte formed in pH 7.7 after 45 days, (e) Spores grown in 30 g/L of sucrose, (f) Heart shaped gametophyte growth in 50 g/L of sucrose after 45 days.

et al., 2010). The influence of different hormones such as BAP, kinetin and GA_3 on spore germination and their gametophytes growth in various concentrations (1-5 mg/L) were studied. All hormonal concentrations significantly increased the percentage of spore germination. The cytokinin (BAP) showed spore sprouting percentages varying from 76 to 90.66% with the absence of rhizoids. The highest sprouting rate (90.66%) was achieved in 4 mg/l of BAP. The spore germination percentages were slightly reduced at 2 mg/L (80.66%), 3 mg/L (82.33%) and 5 mg/L (80.33%) of BAP (Table 3).

When compared to BAP, the Kinetin (KIN) showed minimum spore germination rate ranging from 63.33 to 70.66%. Maximum germination rate (70.66%) was obtained at 4 mg/L of KIN and other concentrations of KIN showed only least differences in their germination rates (1 mg, 63.33%; 2 mg, 63.66%; 3 mg, 64% and 5 mg, 65.33%). Among five concentrations of three hormones used in the study, the rhizoid was emerged only in GA_3 at 3 mg/L (1.66%), 4 mg/L (3.66%) and 5 mg/L (2%). Moreover, all concentrations of GA_3 appreciably increased the germination of *P. tripartita* spores (Figure 2). The similar results in germination rate with least difference were noticed at 2 mg/L (89%), 3 mg/L (90%) and 5 mg/L (91.33%), correspondingly. Among the three hormones, highest spore sprouting rate (95.33%) was observed in 4 mg/L of GA_3. Gibberellins have been used for breaking dormancy in numerous

Table 3. Effects of plant growth hormones on spore germination of *P. tripartita* Sw. after two weeks.

Hormone (mg/L)	Spore germination (%)	Rhizoid formation (%)
BAP		
1	76.00 ± 2.30^{b}	-
2	80.66 ± 5.60^{ab}	-
3	82.33 ± 2.02^{ab}	-
4	90.66 ± 1.45^{a}	-
5	80.33 ± 2.60^{ab}	-
KIN		
1	63.33 ± 3.17^{a}	-
2	63.66 ± 2.40^{a}	-
3	64.00 ± 2.08^{a}	-
4	70.66 ± 1.20^{a}	-
5	65.33 ± 2.02^{a}	-
GA_3		
1	83.00 ± 2.08^{c}	-
2	89.00 ± 1.15^{b}	-
3	90.00 ± 0.57^{ab}	1.66 ± 0.88^{ab}
4	95.33 ± 1.45^{a}	3.66 ± 0.88^{a}
5	91.33 ± 2.40^{ab}	2.00 ± 0.57^{ab}

Data are shown as mean±SE of triplicate values. Each replication consists of hundred spores. Means followed by the same letter within columns are not significantly different at P≤ 0.05 by Duncan's multiple range test (DMRT).

plant species and optimum concentrations accelerating germination depends on species (Sari et al., 1999). The solution containing GA_3 increased the spore germination of *Alsophila spinulosa* (Chen et al., 1991).

Effect of hormones on gametophyte growth

The high rate of gametophyte growth (516.60 µm length and 945.45 µm width) was achieved in 4 mg/L of BAP. In both 2 and 3 mg/L of BAP, moderate growth were observed, in which 354.40 µm of gametophyte length with 287.85 µm of width and 351.07 µm of length with 645.48 µm of width were recorded, respectively. As the BAP hormone concentration increases from 1 to 4 mg/L the growth rate was also increased but, at higher concentration (5 mg/L), the growth rate was rapidly reduced. There was no rhizoidal formation in the gametophytes at 1-3 mg/L of BAP culture condition and also at 5 mg/L of BAP only 324.40 µm of length and 224.41 µm with least number of rhizoid and their length were noticed. Among five concentrations of Kinetin, maximum rate of protonema length (593.27 µm), width (895.46 µm), rhizhoid mean number (3.33) and its length (125.53 µm) were recorded at 3 mg/L (Table 4; Figure 2). There were no rhizhoid formations in the other concentrations of Kinetin. The minimal lengths (335.52 and 307.74 µm) and widths (217.75 and 267.75 µm) of gametophyte growths were observed in both 4 and 5 mg/L of kinetin, respectively.

Rhizoids were noticed in all the concentrations of GA_3. The highest length of rhizhoid (787.69 µm) with maximum rate of gametophyte length (307.74 µm) and width (834.36 µm) were noticed in 1 mg/L of GA_3. The increased concentrations of GA_3 gradually reduced the growth of gametophyte. At both 2 and 3 mg/L of GA_3, moderate lengths (298.85 µm, 261.08 µm), widths (811.02 µm, 377.73 µm) with highest rhizoid numbers (12.30, 13.10) and its lengths (746.58 µm, 754.36 µm) were noticed. Lowest lengths (231.08 and 226.63 µm) and widths (474.39 and 488.83 µm) were noticed with 514.38 and 493.28 µm of rhizhoidal lengths at both 4 and 5 mg/L of GA_3. However, the lower concentration of GA_3 influenced overall morphological growth of gametophytes. Earlier studies also reported the influence of GA_3 on the gametophyte growth of *Lygodium japonicum* and *Blechnum spicant* (Swami and Raghavan, 1980; Fernandez et al., 1997c) and the plant growth regulators such as indol-3-acetic acid (IAA), BAP, gibberellins (GA_3 and GA_{4+7}) on the growth and sexual organ development in spore derived gametophytes of *Blechnum spicant* was also reported (Menendez et al., 2006). Chia and Raghavan (1982) found that GA_3 influenced the gametophyte growth of *Mohria caffrorum* than KIN hormone.

Even though Pteridaceae is a large family, influence of hormones, pH and carbohydrate source on their gametophyte growth are awfully limited. However, *in vitro* culture of *Pteris tripartita* spore summarizes that their spore germination is *Vittaria* type and followed by *Ceratopteris* type of prothallial development. The thalloid adult prothallus was cordate with broad wings, growing very fast and also with a distinct cushion. Rhizoids are nearly hyaline or pale brown that distributed in the lower surface of the cushion with thin cell walls. The adult prothallus is naked and further developments of gametangia are of the common leptosporangiate-type in which antheridia are formed from early development stages of the prothallus. The cap cell becomes loose and pushed off and finally releasing the spermatozoids. The neck of the archegonia is elongated and curving away from the apex of the prothallus (Nayar and Kaur, 1971). The present result agree with former observations on *Vittaria* type of spore germinations which was also reported in *Pteris vittata*, *P. multifida*, *P. wallichiana*, *P. cretica, P. ensiformis, P. inermis, P. fauriei, P. excelsa, P. finotii, Pleopeltis astrolepis*, *P. crassinervata*, *P. macrocarpa*, *P. polylepis, P. revoluta*, *Neocheiropteris palmatopedate, Neottopteris nidus*, *Blechnum appendiculatum*, *B. falciforme*, *B. gracile, B. occidentale, B. polypodioides*, *B. schiedeanum*, *B. serrulatum, B. caudatum* and nine species of *Callipteris* (Reyes Jaramillo

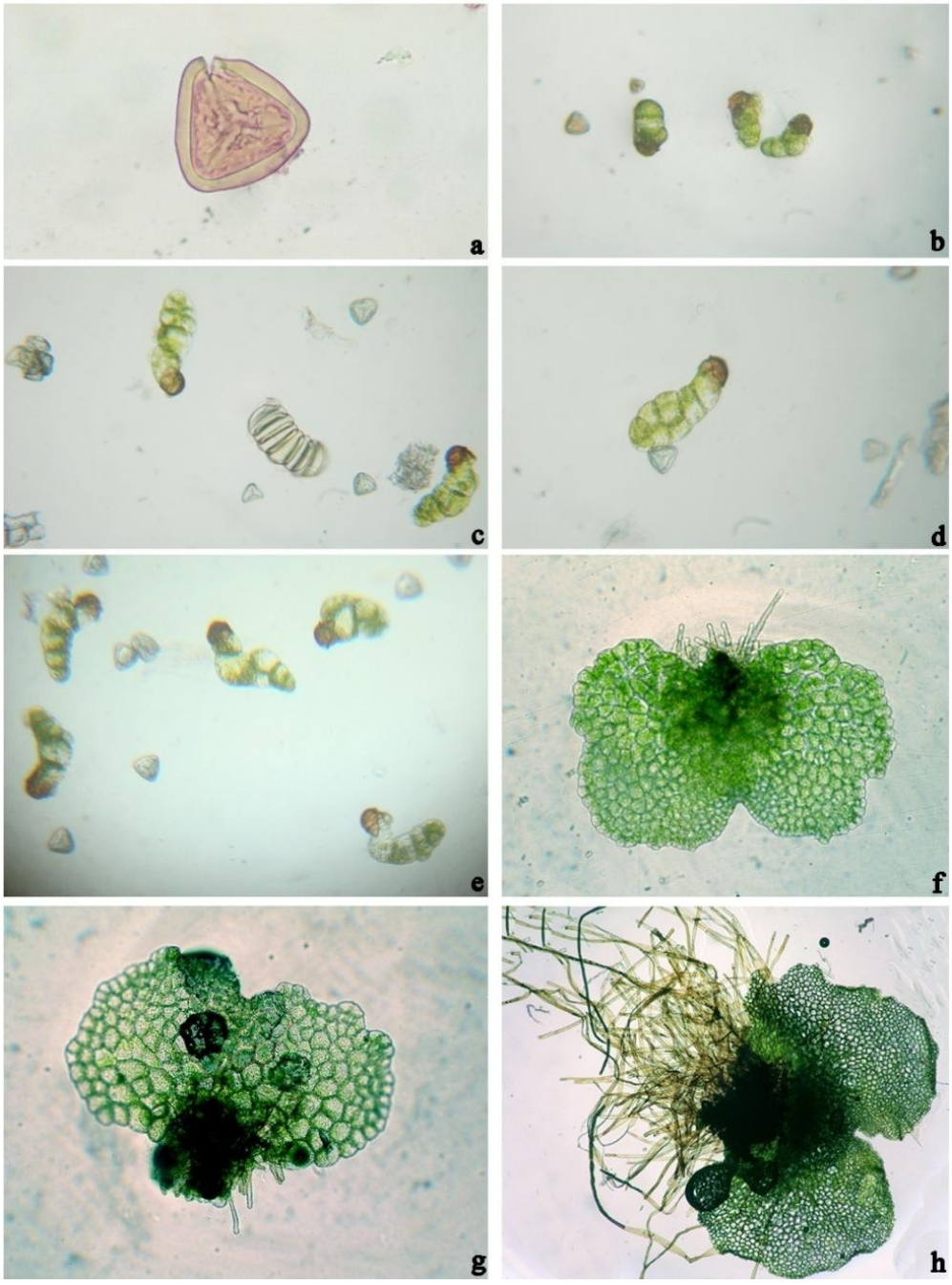

Figure 2. Effect of plant growth hormones on spore sprouting and gametophyte growth of *Pteris tripartita* Sw. (a) Spore, (b) Spore germination in 3 mg/L of KIN, (c) Germination in 3 mg/L of GA_3, (d) Protonemal development at 4 mg/L of BAP, (e) Protonemal cell at 4 mg/L of GA_3, (f) Gametophyte growth in 4 mg/L of BAP, (g) Heart shaped gametophyte at 3 mg/L of KIN (h) Gametophyte growth in 1 mg/L of GA_3.

et al. 2003; Pacheco and Riba, 2003; Zhang et al. 2008; Deng et al. 2009; Zhang, 2009; Mendoza-ruiz and Perez-garcia 2009; Tanco et al. 2009; Shen et al. 2009; Martinez 2010). *Ceratopteris* type of prothalli development was also seen in *Pteris vittata*, *P. multifida*, *P. wallichiana*, *P. finotii*, *P. excelsa*, *P. cretica*, *P. ensiformis*, *P. angusta*, *P. Mexicana* and *Neottopteris nidus* (Reyes Jaramillo et al., 2003; Zhang et al., 2008; Zhang, 2009; Martinez, 2010). However, growth hormones also considerably induced gametophyte morphological growths of *P. tripartita* on their length, width ratio, rhizhoidal and cell number.

Table 4. Effects of plant growth hormones on gametophytic growth of *P. tripartita* Sw. after 45 days.

Hormones (mg/L)	Gametophyte length (µm)	Gametophyte width (µm)	Rhizoidal number	Rhizoidal length (µm)	Gametophyte shape
BAP					
1	292.19 ± 11.60^{d}	246.63 ± 3.33^{d}	-	-	Heart
2	354.40 ± 4.00^{b}	287.85 ± 21.56^{c}	-	-	Heart
3	351.07 ± 7.78^{b}	645.48 ± 1.11^{b}	-	-	Heart
4	516.60 ± 3.33^{a}	945.45 ± 14.69^{a}	3.53 ± 0.88^{a}	108.87 ± 17.46^{a}	Heart
5	324.40 ± 2.22^{c}	224.41 ± 4.00^{d}	0.30 ± 0.30^{b}	8.88 ± 8.88^{b}	Heart
Kinetin					
1	176.64 ± 20.09^{d}	221.08 ± 1.11^{b}	-	-	Spatulate
2	261.08 ± 9.87^{c}	197.75 ± 13.51^{b}	-	-	Spatulate
3	593.27 ± 24.56^{a}	895.46 ± 64.58^{a}	3.33 ± 1.49^{a}	125.53 ± 59.00^{a}	Heart
4	335.52 ± 6.75^{b}	217.75 ± 2.93^{b}	-	-	Spatulate
5	307.74 ± 14.94^{bc}	267.75 ± 28.04^{b}	-	-	Spatulate
GA_3					
1	307.74 ± 4.00^{a}	834.36 ± 17.46^{a}	12.83 ± 1.27^{a}	787.69 ± 4.00^{a}	Heart
2	298.85 ± 7.28^{a}	811.02 ± 9.09^{a}	12.30 ± 0.40^{a}	746.58 ± 6.93^{a}	Heart
3	261.08 ± 6.75^{b}	377.73 ± 85.89^{b}	13.10 ± 0.56^{a}	754.36 ± 27.50^{a}	Heart
4	231.08 ± 1.11^{c}	474.39 ± 5.87^{b}	11.00 ± 0.55^{b}	514.38 ± 6.18^{b}	Heart
5	226.63 ± 3.33^{c}	488.83 ± 4.00^{b}	9.43 ± 0.24^{b}	493.28 ± 28^{b}	Heart

Data are shown as Mean±SE of three replications. Each replication consists of ten gametophytes. Means followed by the same letter within columns are not significantly different at P≤ 0.05 by Duncan's multiple range test (DMRT).

Conclusion

Spores of *P. tripartita* were successfully germinated with significant rates which further developed into normal young gametophytes. Various concentrations of sucrose, pH and plant growth hormones factors either enhance or inhibit the spore germination rate and their gametophyte development. The adult gametophytes of *P. tripartita* were spatulate and cordiform having wide wings without hairs.

Conflict of Interests

There is no conflict of interests.

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Full Length Research Paper

Leaf anatomy of genotypes of banana plant grown under coloured shade nets

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This study aimed to evaluate the effect of spectral light quality on different anatomical features of banana tree plantlets grown under coloured shade nets. Banana plants of five genotypes (Maçã, Thap Maeo, Caipira, BRS Platina and Princesa), obtained from micropropagation, were grown under white, blue, red and black nets, with shade of 50%, in a completely randomized design. After 90 days of acclimatization under nets, the leaves were collected and analyzed anatomically following basic protocol of plant microtechnique. Cultivation under white net provided greater thickness of epidermis cells, hypodermis on the adaxial face and palisade parenchyma; and greater stomatal density on the adaxial face; both the red and white nets, however, increased stomatal density on the abaxial face. The use of white net, during the acclimatization phase, is recommended for cultivation of banana plantlets obtained of micropropagation.

Key words: *Musa* sp., acclimatization, spectral quality, anatomical plasticity.

INTRODUCTION

According to FAO (2012), the banana production worldwide was around 101.9 million tons, and Brazil was responsible for 6.8 million tons, being ranked 5th in the world rankings of the major producing countries. Currently, much of the production of banana plantlets is derived from micropropagation which is a method that promotes the formation of large number of plants in a short time and free of pathogens (Pereira et al., 2005).

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Abbreviations: AdE, Epidermis on the adaxial face; **AbE,** epidermis on the abaxial face; **AdH,** hypodermis of the adaxial face; **AbH,** hypodermis of the abaxial face; **PP,** palisade parenchyma; **SP,** spongy parenchyma; **MR,** midrib; **AdD,** stomatal density of the adaxial face; AbD, stomatal density of the abaxial face; **PD/ED Ad,** polar diameter/equatorial diameter ratio of stomata on the adaxial; **PD/ED Ab,** abaxial faces.

Before being taken to the field, the micropropagated plantlets need a period of acclimatization in the nursery to adapt to the natural environment (Nomura et al., 2012). At this stage, the plants need to be passed to the new climatic conditions gradually; thereby avoiding any type of injury that may occur. The acclimatization phase is extremely important to complete the process of plantlets formation, so it is often considered a limiting factor to the micropropagation process, making it necessary to know the ideal *ex vitro* environment to better adaptation of these plants (Costa et al., 2008). Several researches were reported about the factors that can hold up the nursery phase, such as those related to type of substrate (Nomura et al., 2008) and shading (Scaranari et al., 2009). In this regard, considerable attention has been given to studies that deals with the ability of plant adaptation to light conditions of environment, whether intensity or quality.

It is known that solar radiation is an essential environmental factor for direct or indirect regulation of plant growth and development (Morini and Muleo, 2003). Among the characteristics of radiation, the spectral quality can have significant effects on the morpho-physiological processes of the plant (Schuerger et al., 1997), which can result in changes in plant behavior and the development of different adaptive straregies.

In the literature, we can find numerous reports demonstrating that changes in light quality leads to adaptive changes in plants, such as the rearrangement of the anatomical structure of the leaf to maintain its functionality (Araújo et al, 2009;. Santiago et al, 2001; Hunsche et al., 2010; Solomakhin and Blanke, 2010). This organ has a high degree of anatomical plasticity that contributes to improving the adaptability of plants in different environments (Silva and Nogueira, 2012). The use of coloured shade nets is a way to modify the natural radiation providing an increase in the amount of diffuse light, by combining physical protection with differential refraction of the solar radiation (Henrique et al, 2011; Oren-Shamir et al, 2001).

Thus, studies in environments with different light qualities may assist in obtaining more information about the anatomical changes of the plant, so that the plantlets can be better acclimatized. In this context, this study was performed to evaluate the effect of the spectral quality of light, through the use of coloured nets, on characteristics of leaf anatomy of plantlets of different genotypes of banana plants.

MATERIAL AND METHODS

The experiment was conducted under field conditions from September to December 2010, at Department of Agriculture of Federal University of Lavras. The city of Lavras is located at 21°14'S and 45°00'W GRW with an altitude of 918 m, south of Minas Gerais. According to the Köppen classification, the regional climate is Cwa, but it shows characteristics of Cwb with two distinct seasons defined as follows: a cold and dry season from April to September; and a hot and humid season from October to March (Brazil, 1992).

The plant material used in the experiment was micropropagated banana plantlets of the following cultivar groups: AAB (Maçã and Thap Maeo), AAAB (BRS Platina and Princesa) and AAA (Caipira). The material was donated by Embrapa Cassava and Fruit Culture (Embrapa Mandioca e Fruticultura) and the plants were at their second subculture stage. The plantlets were grown in 24-well polyethylene trays containing a mixture of red latosol (oxisol) and bovine manure (1:1), fertilized according to the recommended method (Ribeiro et al., 1999), and irrigated daily for 90 days.

When the plantlets had approximately 15 cm-high, they were placed inside of structures individually covered with blue and red ChromatiNet-shade nets and white and black shade nets, with 50% of shading. According to the manufacturer, the blue net reduces waves in the range of red and far-red and adds blue waves, and the red net reduces blue, green and yellow waves and adds waves in the range of red and far-red. The white and black nets are not photoconverters; the white net does not interfere on the transmitted light spectrum, and the black net does not change the spectrum, just reduces the irradiance. The average intensity of radiation (measured with USB-850 spectroradiometer Red Tie) inside the nets are shown in Figure 1. The maximum temperature, minimum temperature and photoperiod were varied during the experiment according to the climatic conditions of the city of Lavras.

Initially, plants were also arranged in the condition in full sun, but those plants did not survive in this environment, and then they were discarded from the analysis of the experiment. Thus, we used a 5×4 factorial scheme (five genotypes and four shade nets colors); constituting 20 treatments in a completely randomized design with 5 replicates, each replicate consisted of 4 plants.

The sample collection for anatomical evaluation was performed after 90 days of the treatment regimen. Fully expanded younger leaves were removed from plants of each genotype, fixed in F.A.A.$_{70\%}$ and then stored in 70% ethanol. Paradermal sections of the abaxial and adaxial faces of the leaves were performed by hand using stainless steel blades, while the cross-sections were performed on a microtome table (model LPC). All sections were washed in 1% sodium hypochlorite; paradermal sections were stained with 1.0% safranin, and cross-sections were stained with safrablau solution (1.0% safranin and 0.1% of astra blue at a 7:3 ratio) and then mounted on semi-permanent slides with 50% glycerol (Johansen, 1940). The samples were viewed on an Olympus CX41 microscope coupled to a digital camera (Belcam DIV-3000) and photographed (20 fields for each treatment). Measurements were performed using ImageTool 3.0 software. The following characteristics were assessed: stomatal density, polar diameter/equatorial diameter ratio and thickness of epidermis, hypodermis, palisade parenchyma, spongy parenchyma and midrib. Data were subjected to analysis of variance using SISVAR software (Ferreira, 2011), and the means were compared by the Scott-Knott test at 5% of probability.

RESULTS AND DISCUSSION

Banana tree leaf has a unistratified epidermis on the adaxial and abaxial faces, being classified as amphi-hypostomatic. The mesophyll is characterized as dorso-ventral, showing palisade parenchyma oriented toward the epidermis of the adaxial face just beneath the hypodermis of the adaxial face, and spongy parenchyma is oriented toward hypodermis of abaxial face (Sumardi and Wulandari, 2010). The coloured shade nets promoted

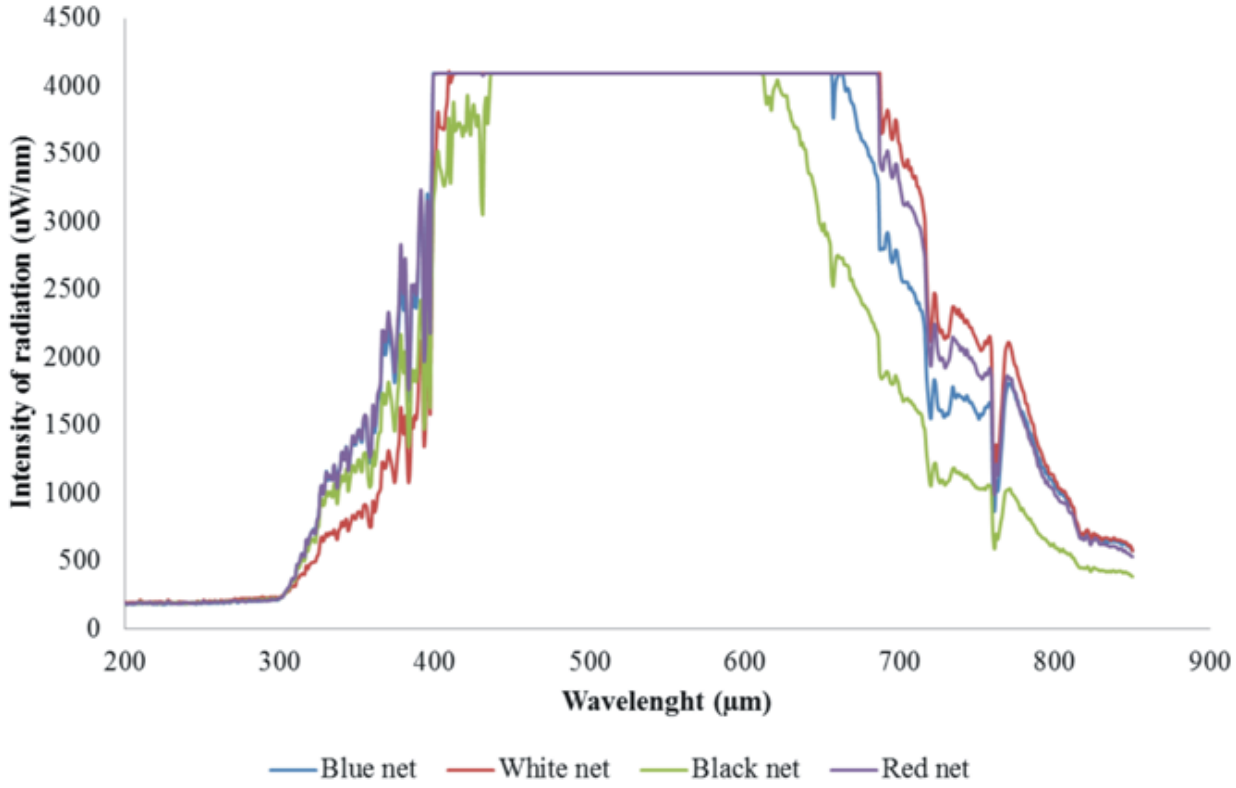

Figure 1. Intensities of radiation of different coloured shade nets according to wavelenght.

significant diferences in epidermis, hypodermis, palisade parenchyma, spongy parenchyma and midrib, as well as in stomatal features (Table 1).

Plants grown under white net showed greater size of epidermal cells on the adaxial face, except for the Caipira genotype which had greater thickness of epidermal cells when grown under red net (Table 2). When considering the epidermis of the abaxial face, the Maçã and Thap Maeo genotypes exhibited greater thickness under white net, while BRS Platina and Princesa genotypes were more prominent under blue net. This behavior was expected, since the same ploidy genotypes tend to exhibit similar behavior.

According to Sumardi and Wulandari (2010), differences in ploidy affect some phenotypic characteristics, with alterations that can often be assessed through observation of plant anatomy. In this study, under all nets, the size of the epidermal cells on the adaxial face was greater than on the cells of the abaxial face. According to Taiz and Zeiger (2004), the epidermis is typically transparent to visible light, and the individual cells are often convex. Convex epidermal cells can act as lenses and focus light so that the amount reaching some of the chloroplasts can be many times greater than the amount of ambient light. In this study, the epidermal cells also have convex shape; however, depending on net used, these cells are larger in size, which increases the concavity of the cell. Hence less light will reach the chloroplasts and the photosynthetic apparatus will be better protected against damage by excess light, being a form of anatomical plasticity of the epidermis. Costa et al. (2010) found no significant difference for thickness of the epidermis on the adaxial face to species *Ocimum selloi*, however, the authors observed greater values for the epidermis on the adaxial face under blue net compared to the red net. The same was observed for the abaxial face.

Also in relation to anatomical aspects of the epidermis, we evaluated the stomatal density and the polar diameter/equatorial diameter ratio of the stomata (Table 3), which are considered as features of high plasticity when plants are cultivated in environments with differences in spectral quality. On the adaxial face, the highest stomatal densities were found under white net, whereas on the abaxial face, greater densities were obtained under white net for Maçã and BRS Platina genotypes, and under red net for Thap Maeo, Caipira and Princesa genotypes.

Similar results were found by Martins et al. (2009) who reported that the cultivation of clove basil (*Ocimum gratissimum* L.) under red net promoted greater stomatal densities on the abaxial face, while adaxially, the cultivation without photoconverter nets was responsible for the greater density. Studies show that greater stomatal density can be found when leaves are exposed

Table 1. Summary of the analysis of variance for studied anatomical features.

SV	DF	Mean square										
		AdE	AbE	AdH	AbH	PP	SP	MR	AdD	AbD	PD/ED Ad	PD/ED Ab
Genotype (G)	4	80.396*	105.212*	4494.771*	2063.043*	2020.738*	1421.262*	254408.835*	118.961*	4706.060*	0.338ns	0.393*
Net (N)	3	15.531^{ns}	8.158ns	3070.403*	386.626*	1336.666*	930.510*	559803.348*	516.776*	19410.676*	0.915*	3.869*
G x N	12	32.332*	24.373*	636.077*	298.901*	207.519*	268.298*	101441.688*	44.346*	4916.080*	0.865*	0.687*
Error	380	10.164	7.253	74.616	68.797	48.546	46.423	4303.021	8.629	86.307	0.153	0.134
CV(%)		19.57	18.38	13.90	16.46	10.57	15.11	9.11	30.76	16.81	17.34	17.46
Average		16.29	14.65	62.12	50.40	65.94	45.11	720.29	9.55	55.28	2.25	2.09

* = significant (Scott-Knott test, 5% of probability); ns = non-significant. SV = Source of variation; DF = Degrees of freedom; CV = coefficient of variation; AdE, Epidermis on the adaxial face; AbE epidermis on the abaxial face; AdH, hypodermis of the adaxial face; AbH, hypodermis of the abaxial face; PP, palisade parenchyma; SP, spongy parenchyma; MR, midrib; AdD, stomatal density of the adaxial face; AbD, stomatal density of the abaxial face; PD/ED Ad, polar diameter/equatorial diameter ratio of stomata on the adaxial; PD/ED, Ab abaxial faces.

Table 2. Biometric measurements of leaves of five banana genotypes grown under different qualities of radiation.

Genotype	Shade net							
	Adaxial epidermis (µm)				Adaxial hypodermis (µm)			
	B	W	Bk	R	B	W	Bk	R
Maçã	16.32^{aB}	18.79^{aA}	16.90^{aB}	14.57^{cC}	58.65^{bB}	71.98^{aA}	53.67^{bC}	60.82^{bB}
Thap Maeo	15.76^{aA}	13.92^{bA}	14.42^{aA}	14.51^{cA}	53.01^{cB}	61.00^{bA}	55.75^{bB}	51.94^{cB}
Caipira	16.70^{aB}	17.27^{aB}	16.30^{aB}	19.08^{aA}	63.23^{bB}	75.77^{aA}	54.91^{bC}	53.60^{cC}
BRS Platina	17.26^{aA}	15.80^{bA}	16.69^{aA}	16.70^{bA}	83.68^{aA}	70.75^{aC}	67.59^{aC}	76.58^{aB}
Princesa	16.43^{aB}	17.97^{aA}	15.39^{aB}	15.05^{cB}	59.32^{bB}	66.15^{bA}	49.47^{bC}	54.66^{cC}
CV (%)	19.57				13.90			
	Abaxial epidermis (µm)				Abaxial hypodermis (µm)			
	B	W	Bk	R	B	W	Bk	R
Maçã	14.32^{bB}	16.65^{aA}	15.02^{aB}	13.83^{aB}	58.41^{aA}	48.99^{bB}	54.82^{aA}	56.24^{aA}
Thap Maeo	12.65^{cA}	12.26^{cA}	13.18^{bA}	13.34^{aA}	43.18^{cA}	42.23^{cA}	44.30^{bA}	46.33^{bA}
Caipira	14.54^{bB}	16.73^{aA}	15.93^{aA}	14.45^{aB}	50.79^{bB}	57.36^{aA}	52.98^{aA}	46.39^{bB}
BRS Platina	17.12^{aA}	14.70^{bB}	15.74^{aB}	15.48^{aB}	62.80^{aA}	50.83^{bB}	53.93^{aB}	53.84^{aB}
Princesa	15.79^{aA}	14.00^{bB}	13.06^{bB}	14.30^{aB}	50.80^{bA}	43.48^{cB}	43.48^{bB}	46.92^{bA}
CV (%)	18.38				16.46			

Means followed by the same lowercase letter in the column and uppercase letter in the row for each variable do not differ by the Scott-Knott test at 5% probability. B, Blue; W, white; Bk, black; R, red.

Table 3. Stomatal features of leaves of five banana genotypes grown under different qualities of radiation.

Genotype	Shade net							
	Adaxial density (stomata/mm^2)				Adaxial PD/ED			
	B	W	Bk	R	B	W	Bk	R
Maçã	10^{aA}	12^{cA}	8^{cB}	8^{aB}	1.83^{dC}	2.52^{aA}	2.06^{bB}	2.17^{aB}
Thap Maeo	9^{aC}	15^{aA}	11^{aB}	10^{aB}	2.28^{bA}	2.17^{bA}	2.36^{aA}	2.23^{aA}
Caipira	7^{bB}	10^{cA}	6^{dB}	9^{aA}	2.08^{cB}	2.49^{aA}	2.29^{aB}	2.19^{aB}
BRS Platina	7^{bC}	15^{aA}	10^{bB}	9^{aB}	2.63^{aA}	2.51^{aA}	1.98^{bB}	2.17^{aB}
Princesa	6^{bC}	13^{bA}	9^{bB}	8^{aB}	2.25^{bA}	2.28^{bA}	2.20^{aA}	2.40^{aA}
CV (%)	30.76				17.34			
	Abaxial density (stomata/mm^2)				Abaxial PD/ED			
	B	W	Bk	R	B	W	Bk	R
Maçã	45^{bB}	58^{aA}	47^{aB}	44^{dB}	1.95^{bB}	2.31^{aA}	1.90^{aB}	2.27^{bA}
Thap Maeo	51^{aB}	49^{bB}	47^{aB}	109^{aA}	1.93^{bC}	2.22^{aB}	2.00^{aC}	2.70^{aA}
Caipira	35^{cD}	60^{aB}	53^{aC}	83^{cA}	1.89^{bA}	2.19^{aB}	1.91^{aA}	2.17^{bB}
BRS Platina	43^{bB}	49^{bA}	49^{aA}	43^{dB}	2.17^{aA}	2.31^{aA}	1.93^{aB}	1.94^{cB}
Princesa	46^{bC}	53^{bB}	44^{aC}	97^{bA}	1.77^{bB}	2.03^{aB}	1.88^{aB}	2.50^{aA}
CV (%)	16.81				17.46			

Means followed by the same lowercase letter in the column and uppercase letter in the row for each variable do not differ by the Scott-Knott test at 5% probability. B, Blue; W, white; Bk, black; R, red.

to high irradiance, giving indications of better control of stomatal conductance, which will reduce water loss through transpiration (Rossatto et al., 2009). Variations in stomatal density on both face of the leaves show the anatomical plasticity of banana genotypes, depending on the growth environment.

Generally, the increase in stomatal density is correlated with a greater stomatal conductance, thus minimizing harmful effects on photosynthesis under different growing conditions (Lima Júnior et al., 2006), and to increased CO_2 uptake, which enables higher photosynthetic rates (Niinemets and Tenhunen, 1997).

The cultivation of plants under white net favored increase of the polar diameter/equatorial diameter ratio (PD/ED) of stomata on the adaxial face of epidermis, while on the abaxial face, different behaviors were observed depending on the genotype (Table 3). The PD/ED ratio of stomata is a marker of the functionality of this epidermal attachment. According to Khan et al. (2002), PD/ED ratio is related to the shape of the guard cells, which constitutes a key particularity for the functioning of stomata, with elliptical shapes (higher PD/ED ratio) being characteristic of functional stomata and rounded shapes (lower PD/ED ratio) characteristic of stomata with abnormal functioning. Variations in functionality highlight the anatomical plasticity of banana leaves of various genotypes in the face of different cultivation environments. Changes in the size and frequency of stomata show the ability that the plants have to rearrange these structures of the epidermis in response to environmental changes, so that there is greater activity of stomata in gas exchange and transpiration, appropriately (Rossatto et al., 2009).

When considering the thickness of the hypodermis, we found different behaviors among the genotypes under the different coloured nets (Table 2). The white net was responsible for the greatest values of thickness of hypodermis on the adaxial face, except for the BRS Platina genotype which experienced its best results hypodermis under blue net. The hypodermis is important in protecting the palisade parenchyma from excess solar radiation, especially during periods of peak

Table 4. Parenchymas thickness of leaves of five banana genotypes grown under different qualities of radiation.

Genotype	Shade net							
	Palisade parenchyma (µm)				Spongy parenchyma (µm)			
	B	W	Bk	R	B	W	Bk	R
Maçã	56.10^{bB}	64.17^{cA}	58.30^{bB}	59.99^{cB}	40.92^{cA}	40.72^{cA}	43.01^{cA}	36.40^{cB}
Thap Maeo	63.49^{aB}	73.60^{bA}	60.95^{bB}	65.61^{bB}	38.90^{cB}	37.96^{cB}	44.60^{cA}	45.19^{aA}
Caipira	57.37^{bC}	64.19^{cB}	69.01^{aA}	60.36^{cC}	41.94^{cB}	43.79^{bB}	59.30^{aA}	42.04^{bB}
BRS Platina	67.63^{aB}	78.29^{aA}	71.93^{aB}	71.79^{aB}	50.66^{aA}	48.92^{aA}	53.71^{bA}	49.79^{aA}
Princesa	63.54^{aB}	72.40^{bA}	66.80^{aB}	73.47^{aA}	45.34^{bA}	43.78^{bA}	47.51^{cA}	47.69^{aA}
CV (%)	10.57				15.11			

Means followed by the same lowercase letter in the column and uppercase letter in the row for each variable do not differ by the Scott-Knott test at 5% probability. B, Blue; W, white; Bk, black; R, red.

incidence of light on leaves (Boeger et al., 2007; Verdaguer et al.,2012). We presume that the white net shade provides a cooler and more humid environment because it reflects all wavelengths of light. Moreover, according to manufacturer, this net works as the full-sun condition, only serving to protect against insects. When considering the thickness of the abaxial hypodermis, the genotypes of the same ploidy showed similar behavior, in order that Maçã and Thap Maeo genotypes exhibited no differences between the nets, BRS Platina and Princesa genotypes had the thickest hypodermis under blue net, and Caipira genotype showed better results under white and black nets. According to Castro et al. (2009), the hypodermis shows no relevant photosynthetic activity, however, it can accumulate water, which is then available in photosynthetic processes.

In the present study, the thickness of the palisade parenchyma was always greater than the spongy parenchyma's (Table 4). All genotypes, with the exception of the Caipira genotype, showed thicker palisade parenchyma when grown under white shade net. When considering the thickness of the spongy parenchyma, we also found that the responses varied for all genotypes depending on the net used. The BRS Platina and Princesa genotypes leaves exhibited no significant differences in the parenchyma thickness. The Caipira genotype leaves showed greater development of spongy parenchyma when grown under black net. The Maçã genotype exhibited similar behavior under blue, white and black net, and the Thap Maeo genotype leaves had thicker spongy parenchyma under black and red nets. Spongy parenchyma has a large amount of intercellular spaces, which are crucial for gas storage. A considerable amount of gases can enter the mesophyll and be retained in the intercellular spaces; CO_2, in particular, can then be fixed by the palisade parenchyma (Castro et al., 2009). Therefore, the increase in spongy parenchyma contributes to an increase in the available area for CO_2 absorption (with subsequent assimilation); this increased area is often larger than that of the external surface of the leaf.

According to Appezzato-da-Glória and Carmello-Guerreiro (2006), the palisade parenchyma and the spongy parenchyma are considered as assimilators parenchymas which have photosynthetic activity. This fact is due to the presence of chloroplasts which convert light energy into chemical energy, and store it in the carbohydrates form. Thus, further development of these tissues can result in a higher photosynthetic efficiency, which can contribute to the rapid growth of the plant during the acclimatization phase. It is expected that thicker palisade parenchyma result in greater photosynthetic rates (Bolhar-Vordenkampf and Draxler, 1993), which are essential to the growth and development of plants. Usually, the palisade parenchyma is more developed than the spongy parenchyma, which optimizes photosynthesis and decreases the intercellular spaces of the spongy parenchyma, thus reducing the availability of water vapor subject to transpiration (Castro et al., 2009). Costa et al. (2010) found that the thickness of palisade parenchyma of leaves of *Ocimum selloi* (pepper basil) did not change when plants were grown under blue and red shade nets; however, the spongy parenchyma was thicker under these conditions. In the present study, the spongy parenchyma showed different responses depending on the genotype analyzed. Dimassi-Theriou and Bosabalidis (1997) found that the thickness of the palisade parenchyma increased when kiwi plants were subjected to higher solar radiation. In the present study, for most genotypes, the palisade parenchyma was thicker under the white net. White shade nets do not affect the spectrum of irradiated light; in other words, they produce a change in the quantity but not the quality of radiation. In cooler environments, thickening of the palisade parenchyma is favored, so as to enable increased photosynthetic activity (Boeger et al., 2007; Haberlandt, 1990).

In this study, the black net was responsible for providing the greatest thicknesses of midrib for all genotypes, with the exception of Thap Maeo genotype

Table 5. Midrib thickness of leaves of five banana genotypes grown under different qualities of radiation.

Genotype	Midrib (µm)			
	B	W	Bk	R
Maçã	638.08^{bC}	794.35^{bB}	875.06^{aA}	796.13^{bB}
Thap Maeo	710.93^{aB}	577.37^{dC}	705.46^{bB}	767.03^{bA}
Caipira	499.19^{dD}	643.95^{cB}	751.48^{bA}	588.40^{dC}
BRS Platina	626.40^{bC}	864.72^{aA}	889.14^{aA}	699.23^{cB}
Princesa	569.42^{cC}	684.86^{cB}	866.28^{aA}	858.30^{aA}
CV (%)	9.11			

Means followed by the same lowercase letter in the column and uppercase letter in the row for each variable do not differ by the Scott-Knott test at 5% probability. B, Blue; W, white; Bk, black; R, red.

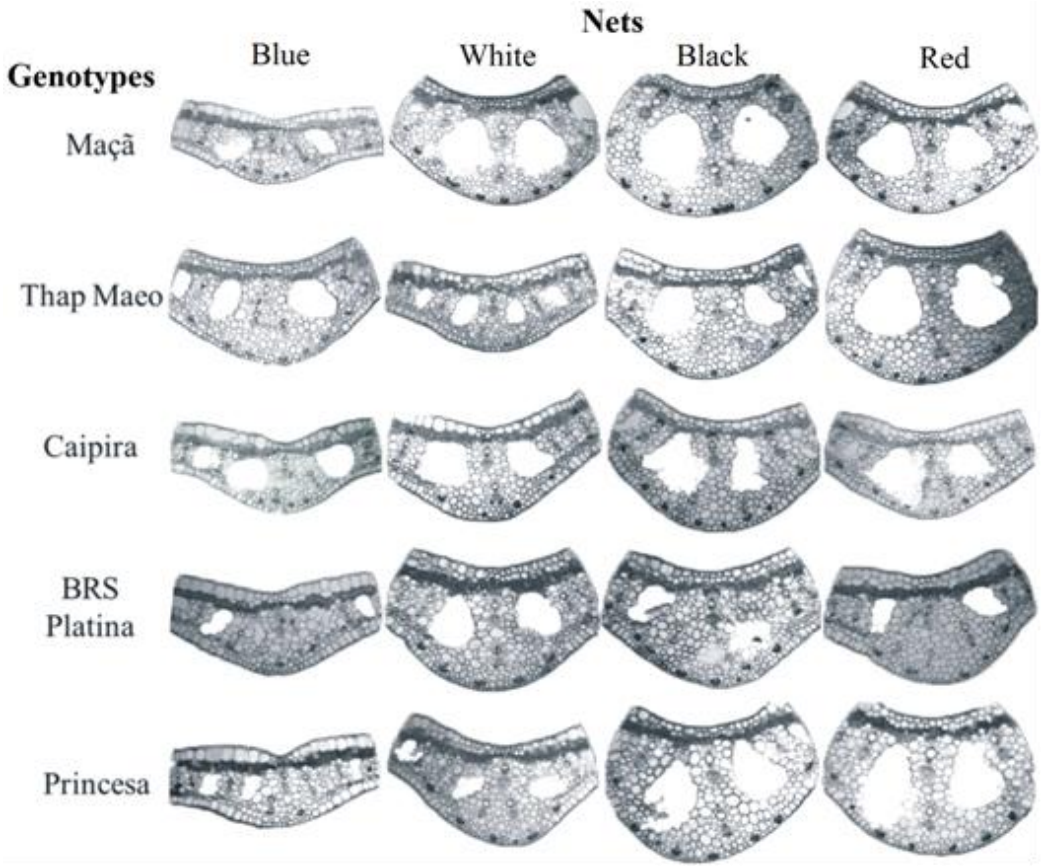

Figure 2. Photomicrographs of banana leaves showing the plasticity of midrib of five genotypes grown under environments with different light qualities. Bar = 200 µm.

that exhibited the greatest values for that feature under red net (Table 5).

Thicker midribs indicate adaptation to stressful conditions, such as situations of low irradiance. Figure 2 shows clearly the plasticity of the midrib of the banana leaves under different radiation qualities.

Conclusion

Banana leaves exhibit anatomical plasticity in response to changes in the spectrum of radiation. This plasticity can vary depending on the cultivated genotype; plants belonging to the same genomic group do not respond

uniformly to changes in light quality. Cultivation under white net provided greater thickness of epidermal cells, hypodermis on the adaxial face, and palisade parenchyma and greater stomatal density on the adaxial face; both red and white nets, however, promoted an increase of stomatal density on the abaxial face. Thus, it may be recommended to use white net during the acclimatization phase for cultivation of micropropagated banana plantlets due to the development of anatomical features more favorable to the plant growth.

Conflict of Interests

The author(s) have not declared any conflict of interests

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Full Length Research Paper

Phytochemical screening and antimicrobial activity of apiary honey produced by honey bee (*Apis mellifera*) on clinical strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*

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Honey produced by honeybee (*Apis mellifera*) which is used in herbal medicine was examined for its chemical constituents and antimicrobial activity. The phytochemical analysis of honey showed the presence of alkaloids, flavonoids, saponins, steroids, reducing sugar and glycosides. Antimicrobial activity of honey on fresh hospital isolates: *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* obtained from Glanson Medical Laboratory Awka were determined using well diffusion method. The result shows that the honey produced by *mellifera* has strong antimicrobial activity against *E. coli* and *S. aureus* but not against *C. albicans*. The result obtained shows that the honey produced a zone of clearance of 45 and 34 mm on *S. aureus* and *E. coli*, respectively. The result of minimum inhibitory concentration (MIC) determined on liquid culture was 20%v/v for both *S. aureus* and *E. coli* while the minimum bactericidal concentration (MBC) determination of the sample showed 20% and 30%v/v for *S. aureus* and *E. coli*, respectively. Our result shows that honey, apart from their role as food additives and supplements, may also be utilized as effective and cheap sources of antibacterial agents for the treatments of bacterial infections.

Key words: Apiary honey, *Apis mellifera*, antibacterial activity, minimum inhibitory concentration, minimum bactericidal concentration, clinical isolates.

INTRODUCTION

The use of plant for healing is as ancient and universal as medicine itself. Plants act generally to stimulate and supplement the body's healing forces. They are the natural food for human beings. Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Today, plant materials continue to

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play a major role in primary health care as therapeutic remedies in many developing countries. Plants still continue to be almost the exclusive source of drugs for the majority of the World's population. The antimicrobial activity screening and phytochemical analysis of essential plants has been of great interest in the discovery of drugs effective in the treatment of several diseases (Ainslie, 1999). Herbs have played an important part in our developments. Approximately 25% of all prescription drugs are derived from trees, for example, shrubs of foxglove, morphine and codeine are derived from the opium, and quinine from cinchoma bark etc. The essential differences between herbal and conventional medicine is that; in conventional medicine, the most active constituent is extracted from the plant, synthesized in the laboratory to make drugs while in herbal medicine, the extract from the whole plant are used (W.H.O, 2000).

The scope of herbal medicine is extended to include fungal such as mushroom and bee products like honey as well as animals, shells and certain animal parts. Man has been so blessed in nature. Honey is a natural gift to man from Mother Nature which is made available to us from the mysterious kingdom of the bees, but it is quite unfortunate that we have cared less to know what is contained in this wonderful nature's kits (Akinpelu, 2000).

The resistance of antibiotics against pathogens has triggered research scientists to venture for substitute curatives. It is indeed of paramount importance to unveil new therapies directed at novel targets as budding to alternatives to antibiotics as well as validation of traditional remedies (Jenkins et al., 2011). Plethora of studies has emerged towards natural products in addressing the dearth and limitations of current therapies. One natural food product which has gained great momentum is honey. Honey, a natural product of very high nutritive value is made when the nectar (floral) and sweet deposits from plants (non floral) are gathered, modified and stored in the honeycombs by honeybees of the genera *Apis* and *Meliponini* (Namias, 2003; Al-jabri, 2005). Its composition and quality vary greatly with the botanical source of nectar as well as environmental and climatic conditions. Depending on its quality, honey can contribute to the health and nutritional status of humans. These beneficial actions have been ascribed to its antimicrobial, anti-inflammatory and anti-oxidant potential. Interestingly, honey is gradually receiving attention as a complementary and an alternative source of treatment in modern medicines. It is active against antibiotic-sensitive and antibiotic-resistant strains of micro-organisms and has the potential not to select for further resistant strains (Manyi-Loh et al., 2011).

There are basically two main types of honey, apiary and forest honeys. Honeys produced by the honeybees, *Apis cerana indica* and *Apis mellifera*, in apiaries and collected by the modern extraction method are called apiary honey. They are transparent and free from foreign materials. In contrast, those produced by rock bee, *Apis dorsata*, or from wild nests of *A. cerana indica* in forests and collected by the crude method of squeezing the comb are known as forest honeys. They are turbid owing to the abundance of pollen, wax, brood (bee larvae), parts of bees, and plant materials. It is therefore necessary to filter the honey to separate the suspended particles (Subrahmanyam, 2007).

Researchers round the globe have worked both *in vitro* and *in vivo* to spark the unknown benefits of the inestimable attributes of honey as well as its applications (Cursons, 2010; Irish et al., 2008; Kumari et al., 2010; Zaid et al., 2010). In the modern era, the different biological, chemical and physical properties of honey have revealed several beneficial claims through different techniques. The multi facet properties of honey anchored in the scientific world is regarded as a sweetener, functional food, antioxidant, antimicrobial, antiseptic, prebiotics, probiotics, immunomodulatory, anti-inflammatory, anti-tumor and anti-cancer effect amongst others (Jenkins et al., 2011; Conway et al., 2010; Fauzi et al., 2011). Above and beyond its therapeutic effects or medicinal attributes (Mohapatra et al., 2011; Conti et al., 2007), it is also of potential use as bio-indicators for environmental contamination (Celechovska and Vorlova, 2001). The colour of honey can vary from nearly colourless to dark brown and its consistency can be fluid, viscous or partly to entirely crystallized. The botanical spectrum or the nectar source visited by the honey bees leads to variation in colours, flavours and aroma (C. A. C, 1996).

Honey is well known for its antibacterial activity, which was first reported in 1892. Since ancient times, honey has been used for treatment and prevention of wound infections. With the advent of antibiotics, the clinical application of honey was abandoned in modern Western medicine, though in many cultures it is still used. For all antibiotic classes, including the major last resort drugs, resistance is increasing worldwide (Walsh, 2003; Levy and Marshal, 2004); and even more alarming, very few new antibiotics are being developed. The potent activity of honey against antibiotic-resistant bacteria resulted in renewed interest for its application (Cooper et al., 2002a; Cooper et al., 2002b; Efem et al., 1988). Several honeys have been approved for clinical application. The incomplete knowledge of the antibacterial compounds involved and the variability of antibacterial activity are however major obstacles for applicability of honey in medicine. In recent years, the knowledge on the antibacterial compounds in honey has expanded.

According to the United States National Honey Board (1994) and various international food regulations, honey is a sweet aliment produced by honey bees (*A. mellifera*) and derived from the nectar of flowers. It also stipulates a pure product that does not allow the addition of any other sweetener, but is not limited to water or other substance. Honey gets its sweetness from monosaccharide such as fructose, glucose and has approximately the same

sweetness as that of granulated sugar. Honey is one of the oldest traditional medicines considered to be important in the treatment of respiratory ailment, gastrointestinal infection and various other diseases (W.H.O, 1996). It is being used effectively in dressing of wounds, burns and skin ulcers to reduce pain and odour quickly. The ability of honey to kill microorganisms has been attributed to its high osmotic effect, high acidic nature (pH 3.2 to 4.5), hydrogen peroxide concentration and its phytochemical nature; that is, its content of tetracycline derivatives, peroxide, fatty acids, phenols etc.

The aim of this study was therefore to evaluate the chemical constituents and antimicrobial potential (bacteriostatic and bactericidal effect) of honey produced by honey bees (*A. mellifera*) on *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* strains isolated from wound, feces and vaginal swab of patients.

MATERIALS AND METHODS

Sample collection and mode of identification of pure/original honey

Honey samples were collected from harvesters at Nsukka, Enugu state Nigeria. The samples were confirmed to be honey by conducting several experiments, which included:

i) Dipping a match stick into the honey and striking it: the matches will burn if it is a pure honey and the honey will even act as a fuel while the match is burning.
ii) Dropping some of the sample onto sand: if it is a pure honey, it will not sink immediately.
iii) Dipping a finger into the honey and trying to drop one or two drops on the ground: if it is pure, it will go down like a thread without breaking.
iv) Pouring a small quantity of honey into a cup of water: if pure it will go down to the bottom of the cup without mixing up with the water except if stirred.

These entire tests were done to ascertain that the sample was pure and original honey was used. It was then filtered with a sterile mesh to remove debris and then stored in a sterile bottle before use.

Phytochemical analysis of honey produced by *A. mellifera*

The sample was screened for the following compounds: alkaloids, flavonoids, glycosides, phenols, saponins, tannins and reducing sugar using standard laboratory techniques (Harbonrne, 1992; Sofowara, 1993).

Confirmatory identification of test organisms

The test organisms used in this work were obtained from Glanson Medical Laboratory Awka, Anambra State. The organisms are: *E. coli, S. aureus, C. albicans.* The following biochemical tests were carried out to confirm the identity of the organisms: Gram stain, Catalase test, Oxidase test, Indole test and lactophenol test.

Antimicrobial screening of honey

The antimicrobial activity was tested using the agar well diffusion method (Chung et al., 1990). Nutrient agar was used to study antibacterial susceptibility while Sabouraud dextrose agar was used for antifungal susceptibility test. Twenty - four to 48 h broth cultures of the test organisms was diluted to 10^{-2}. One millilitre of the diluted culture was added to 100 ml of sterile molten Nutrient agar (40 to 45°C) and Sabouraud dextrose agar for the yeast in a 250 ml flask. The content was mixed very well and 20 ml of it poured into each Petri dish and allowed to solidify. A sterile cork borer (a metallic hollow cylinder) was used to create wells in the agar. The wells were aseptically filled with the honey sample using a dropping pipette and the plates incubated at 37°C for 24 h or at 25°C for 72 h for bacteria and fungi, respectively. Zones of inhibition were measured after incubation.

Determination of minimum inhibitory concentration (MIC)

The honey produced by honey bees (*A. mellifera*) was used to determine the MIC on the bacterial organisms in liquid culture. The MIC is the lowest concentration that is able to inhibit any visible bacterial growth on the culture tube (Prescott et al., 2008). The following concentrations of the honey sample; 1, 2.5, 7.5, 10, 15, 20 and 30%v/v corresponding to the following volumes; 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 ml were made in test tube each containing 10 ml of sterile nutrient broth. In each test tube 0.5 ml was added at 24 h culture diluted to 10^{-5}. The tubes were examined for visible growth after 24 h incubation.

Determination of minimum bactericidal concentration (MBC)

MBC is the lowest concentration of the sample that prevents bacteria growth after incubation or required to kill the organism (Prescott et al., 2008). This was obtained by streaking out the samples from the MIC tubes that showed no visible growth on nutrient agar plates. MBC were indicated by failure of the organism to grow on the media plates after 24 h incubation.

RESULTS

Phytochemical analysis of honey produced by honeybees (*A. mellifera*)

The results of the phytochemical analysis show that the honey contains alkaloids, flavonoids, saponins, glycosides, and reducing sugar (Table 1). The intensity of colour change is a semi - quantitative measure of the amount of each chemical present in the sample and is represented in Table 1 by the number of plus signs (+). The results of antimicrobial screening are shown in Table 2.

The sample showed antibacterial activity against *S. aureus* and *E. coli* with zone of inhibition/clearing of 45 and 34 mm in diameter, respectively while there was no inhibition on *C. albicans*.

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration observed to be 20% (2 ml of the sample in 10 ml of broth) for both *S. aureus* and *E. coli*.

Table 1. Phytochemical analysis of apiary honey produced by *Apis mellifera.*

Parameter	Value (Inference)
Alkaloids	++
Flavonoids	+++
Saponins	+
Tannins	-
Phenols	-
Glycosides	+
Reducing sugar	++

+++, Highly present; ++, moderately present; +, slightly present; -, Nil. Antimicrobial screening of honey produced by honeybees (*Apis mellifera*).

Table 2. Zone of inhibition of the honey produced by *Apis mellifera* on the test organisms.

Test organism	Zone of inhibition in diameter (mm)
S. aureus	45
E. coli	34
C. albicans	nil

Table 3. Minimal bacterial concentration (MBC) of the honey (*Apis mellifera*) on the test organisms.

Concentration (%)	Test organism	
	S. aureus	***E. coli***
10	+	+
15	+	+
20	-	+
30	-	-

+, Growth; -, No growth

Minimal bacterial concentration (MBC)

The MBC for *S. aureus* was observed to be 20% while that of *E. coli* was 30%v/v of the sample (Table 3).

DISCUSSION

The result of the phytochemical analysis of honey as shown in Table 1 indicates that alkaloids, flavonoids, glycosides, saponins and reducing sugar are present in the honey sampled here. These classes of compounds are known to possess therapeutic properties against several pathogens and are therefore supporting its traditional use in curing diseases. Saponins detected in honey have been found to be an antibacterial substance on cell wall of many organisms (Harborne, 1992).

Flavonoids help in the healing of wounds and treatment of skin diseases due to their ability to neutralize the acidity of wounds, and inflammation. Plants containing alkaloids are used in the treatment of malaria, cold, and cough (Thomson, 1987). Treatment of heart diseases could be because of flavonoid, saponins and glycosides which stimulate heart, especially saponin that remain within gastrointestinal tract. Some interact directly with dietary cholesterol producing an insoluble complex which prevents the cholesterol from being absorbed. Dietary saponins reduce plasma cholesterol level in primate thus having the potential to lower the risk of coronary heart diseases in humans (Macrae et al*.,* 1993).

Results of the well diffusion test, reported in Table 2 showed that the honey sample has antibacterial activity against both Gram positive and Gram negative organisms but not against yeast cells. This is an indication that honey can be a potential treatment for diseases caused by *S. aureus* and *E. coli.* Similarly, previous work has shown that honey has been used to heal recalcitrant wounds whereby it was found to be effective *in vitro* against a wide range of multi-resistant organisms including methicillin resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and multiresistant *Pseudomonas aeruginosa* (Cooper et al., 2002; George and Cutting, 2007). Our findings are also in agreement with the work of Nzeako and Hamdi (2000). Their study has shown that honey (*A. mellifera*) has an antimicrobial activity against *S. aureus* and *E. coli*. Another study by Kingsley (2000) also reported that honey completely inhibited major wound infectious pathogens such as *Staphylococcus pyogenes* and *S. aureus*. Similarly, a study by Mogessie Ashenafi (1994) reported that tazma mar honey produced by sting-less bees (*Apis mellipodae*) was found to be effective against some food-borne pathogens of humans, including *Staphylococcus typhimurum*, *Staphylococcus enteriticlis* and *E. coli*. The result of our study is consistent with the above studies. Furthermore, Rendel et al. (2001) demonstrated that acidification of wounds speeds healing; this being attributed to low pH increasing the amount of oxygen off load from hemoglobin in the capillaries. Actually, acidification prevents ammonia produced by bacteria metabolism from harming body tissues (Williams et al., 2009).

The honey used in this research did not inhibit the growth of *C. albicans*. This result does not agree with the work of Koc et al. (2009), who in their study demonstrated *in vitro* that honeys from different floral sources in Turkey had antifungal activity at high concentration of 80% v/v against 40 yeast species, including *C. albicans, Candida krusei, Candida. glabrata* and *Trichosoporon* spp. Cutaneous and superficial mycoses like ringworm and athletes foot are found to be responsive to honey (Bansal et al., 2005). This disagreement may be as a result of differences in the experimental conditions.

In this study, the minimum inhibitory concentration (MIC) was observed to be 20% for both *S. aureus* and *E.*

coli. In contrast to this report, Molan (1999) observed that honey produced by honeybees (*A. mellifera*) could inhibit most of the test organisms including *S*. *aureus* and *E*. *coli* at a very low concentration (2.5 to 7.5%v/v). Another study by Molan (2000) reported that the minimum inhibitory concentration (MIC) and the minimum bacteria concentration (MBC) for *E*. *coli* were found to be 7 and 10%, respectively. The variation in the antimicrobial potential of honey used in this present study as compared to the previous studies highlights that the source of the nectar may have contributed to the difference in the antimicrobial activities of honey; that is, the flowers from which bees gathered nectar to produce the honey, since flora source determines many of the attributes of honey; for example flavour, aroma, colour and composition of honey is highly variable as demonstrated by Mogessie (1994). The variation may also be attributed to differences in growth rate of pathogens, nutritional requirement, temperature, inoculums size and the test methods itself (Gaill and Jon, 1995).

The presence of antimicrobial substances as demonstrated by zone of inhibition showed distinctly the efficacy of apiary honey as a medicine for the treatment of ailments caused by *S*. *aureus* and *E*. *coli*.

Conclusion

This work has shown that apiary honey produced by *A. mellifera* has both bacteriostatic and bactericidal activity when tested. Moreover, the pharmacological, standardization and clinical evaluation on the effect of honey are essential before using it as a preventive and curative measure to common diseases related to the test organisms. Therefore, the antibacterial activity of honey produced by *A. mellifera* against clinical bacterial isolates indicates the usefulness of the honey in clinical practice against bacterial but not fungal (*C. albicans*) infections.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Purification, characterization of phytase enzyme from *Lactobacillus plantarum* bacteria and determination of its kinetic properties

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Phytases (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyze the release of phosphate from phytates. Many of the cereal grains, legumes and oilseeds store phosphorus in phytate form. Phytases can be produced by plants, animals and microorganisms. However, the ones with microbial origin are the most promising for commercial uses and biotechnological applications. In this study, phytase enzyme isolation from *Lactobacillus* spp. ATCC strain and its characterization was carried out. Phytase production from bacterial strains was determined by zone production formed around colonies after 48 h of incubation at 30°C in MRS medium. Optimum pH and optimum temperature values of the phytase enzyme that was partially purified by precipitation of ammonium sulphate from *Lactobacillus plantarum*, extracellularly from bacteria put into liquid culture medium, were measured. Optimum activity of the enzyme derived from *L. plantarum* bacterium was at 30°C and pH 6.0. It was observed that *L. plantarum*'s extracellular enzyme maintains its 90% of activity at 10-100°C for 120 min. Effects of certain metal ions on activity of phytase enzyme derived from *L. plantarum* were also investigated. Of these, $CuCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$ and $ZnCl_2$ decreased enzyme activity significantly. $FeCl_2$ increased enzyme activity by 121%. Based on these results, the phytase enzyme of *L. plantarum* is considered suitable for use in many industrial areas, in feed and food industries in particular, due to its thermal stability and resistance to metal ions.

Key words: Purification, characterization, phytase, *Lactobacillus plantarum*.

INTRODUCTION

Phytases (myo-inositol hexakisphosphate phosphohydrolas, EC 3.1.3.8) are enzymes that catalyze phytic acid's (myo-inositol hexaphosphate) hydrolysis reaction to myo-inositol phosphate, inorganic monophosphate and free myo-inositol (Kerovuo, 2000). Phytase enzyme was first found in rice bran (Suzuki et al., 1907) and in the blood of calves (McCollum and Hart, 1908). Then, the presence of phytase enzyme in plants, yeast, fungi and

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microorganisms was identified (Li et al., 2013). Today, bacterial phytases have more alternative uses than other phytase sources, due to their features such as commercial substrate specificity, resistance against metal ions, thermal stability, proteolysis-resistance and high catalytic activity (Konietzyn and Greiner, 2004; El-Toukhy et al., 2013).

Phytases increase the bioavailability of phosphorus contained in the structure of raw feed and cannot be utilized normally by facilitating its digestion. Phytase enzyme breaks down phytic acid in producing fertilizer and paper pulp and hence inhibits the formation of carcinogenic or toxic substances, and helps to decrease environmental pollution caused by phosphorus. In addition, it was also found that plant growth is stimulated when phytase enzyme is added to soil, depending on the increase in the decomposition rate of phytin (Dalal, 1978; Findenegg and Nelemans, 1993).

Phytase enzyme was purified and characterized from several microbial sources, such as *Pseudomanas* spp. (Irving and Cosgrove, 1971), *Bacillus subtilis* (Powar and Jagannathan, 1982), *Bacillus subtattoilis* (Shimizu 1993), *Bacillus amyloliquefaciens* (Kim et al., 1998), *Eschericia coli* (Greiner et al., 1993), *Klebsiella aerogenes* (Tambe et al., 1994), *Klebsiella terrigena* (Greiner et al., 1997), *Klebsiella oxytoca* (Jareonkitmongol et al., 1997), and *Enterobacter* spp. (Yoon et al., 1996).

Lactic acid bacteria (LAB) are Gram-positive, facultative anaerobic, catalase-negative, immobile (with one or two exceptions), cytochrome-free bacteria that do not produce spores except *Sporolactobacillus inulinus*, and produce lactic acid as an end product during carbohydrate fermentation. These bacteria are almost never present in water and soil, however, their various genus and species can be seen in milk, dairy products, dairy farms, plants, plant waste, and intestinal systems of humans, animals and other living organisms; and these bacteria are non-toxic (Hofvendahl et al., 2000; Tungala et al., 2013). *Lactobacillus plantarum* used in our study is a LAB and is an important bacteria in terms of biosecurity.

The aim of this study was to produce phytase enzyme extracellularly from *L. plantarum* to purify partially, and study its certain kinetic properties.

MATERIALS AND METHODS

Microorganism strains and medium used

The lactic acid bacteria *L. plantarum* used in the study were provided by Laboratory of Microbiology, Department of Food Engineering, University of Ataturk. Bacteria seeded into MRS agar first, and activated by incubating for 48 h at 26 to 30°C. Later, a sample taken from a single colony was added to MRS Broth and allowed to incubate for 48 h again. Then, extracellularly obtained crude extract was used in the enzyme studies. During this time, liquid cultures were allowed to stand in the refrigerator (+4°C).

Ammonium sulphate precipitation for phytase enzyme

After centrifugation of bacteria broth at +4°C at 9000 x *g* for 10 min, supernatant and the precipitate were separated. The precipitate was discarded after centrifugation and the supernatant fraction was subjected to ammonium sulfate precipitation. In order to determine the precipitation range of phytase enzyme, ammonium sulphate precipitation was performed at 0-20, 20-40, 40-60 and 60-80% saturations. Precipitates were dissolved in small amount of 0.1 M Tris-HCl buffer (pH 5.5), and phytase activity was determined both in the precipitate and supernatant (Nadaroglu and Tasgin, 2013). Active fractions were dialyzed against the same buffer. Active fractions were pooled and allowed to stand at 4°C.

Measuring enzyme activity

Enzyme activity was determined by using Na-phytate. In short, 0.1 mL enzyme solution and 250 µL Na-fitate were mixed in a vortex and incubated for 10 min at 37°C. Then, the reaction was stopped by adding 500 µL 10% TCA to the medium, and after incubating for 5 min at 90°C, a 500 µL coloring solution was added and allowed to stand for 15 min, then it was centrifuged for 5 min at 3000 x *g*. After centrifugation, absorbant change in the samples was measured spectrophotometrically (PG Instrument T80 Spectrophotometer) at 700 nm against blind sample.

Determining optimum pH value of enzyme

The enzyme activity was measured in the range of pH 2.0-11.0, in order to determine optimum pH of the phytase enzyme purified from *L. plantarum*. 10 mM acetate buffer was used for the pH 2.0-6.0 range, Tris/HCl buffer was used for the pH 6.0-9.0 range, and carbonate buffer was used for the pH 9.0-11.0 range to measure enzyme activity spectrophotometrically for NA-phytate substrate (Figure 1).

To determine the optimum pH of phytase enzyme in the range of pH 2.0-11, Sodium Acetate (pH 11-6.0), Tris (pH 7.0-9.0), and Carbonate (pH 10-2.0) buffers were used, and optimum pH value for Na-phytate substrate was determined spectrophotometrically by using the above-mentioned standard method (Figure 1).

Determining stable pH value of enzyme

Activity measurements were performed in the range of pH 2-11 in order to determine pH stability of the phytase enzyme produced extracellularly and purified from *L. plantarum*. For this purpose, the buffer solutions mentioned above and enzyme solutions that were adjusted for each pH values were put into the reaction medium, and enzyme activity was determined at certain intervals for 10 h and the pH stability of the enzyme was determined (Figure 2).

Determining optimum temperature value of enzyme

For determination of optimum temperature of enzyme activity, the above-mentioned activity assay was used for activity measurements at 10°C intervals ranging from 10 to 100°C (Figure 3).

Determining stable temperature value of enzyme

In order to determine temperature stability of the phytase enzyme purified from *Lactobacillus plantarum* extracellularly, the enzyme solution was allowed to stand at temperature values ranging from 10 to 100°C, and activity measurements were performed at every 15 min for 2 h (Figure 4).

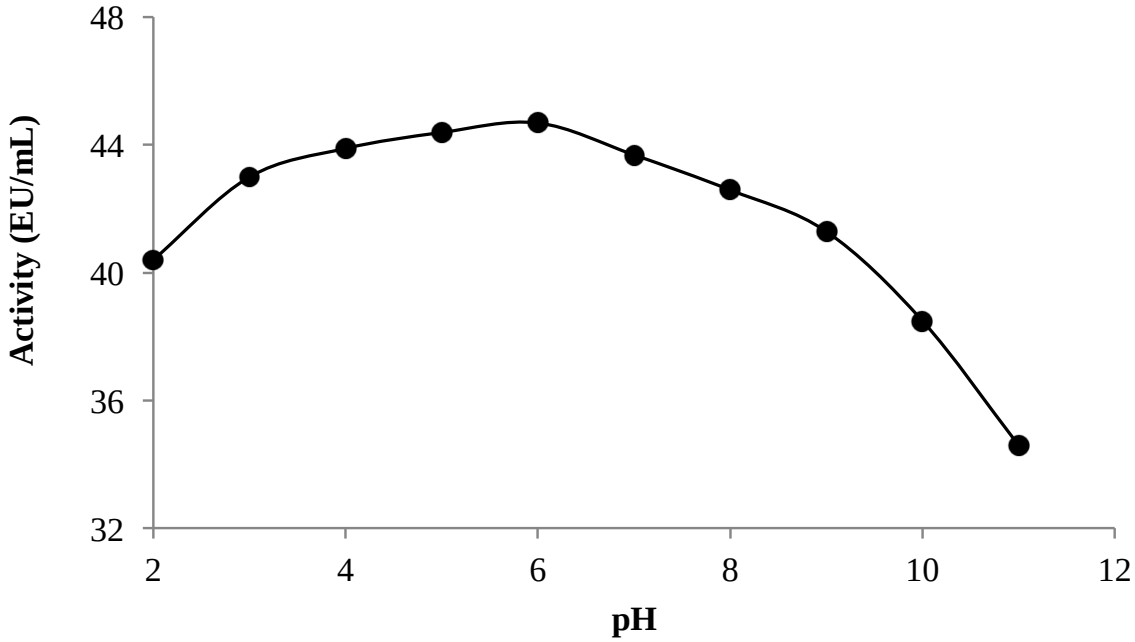

Figure 1. The effect of pH on the activity of purified phytase from *L. plantarum.*

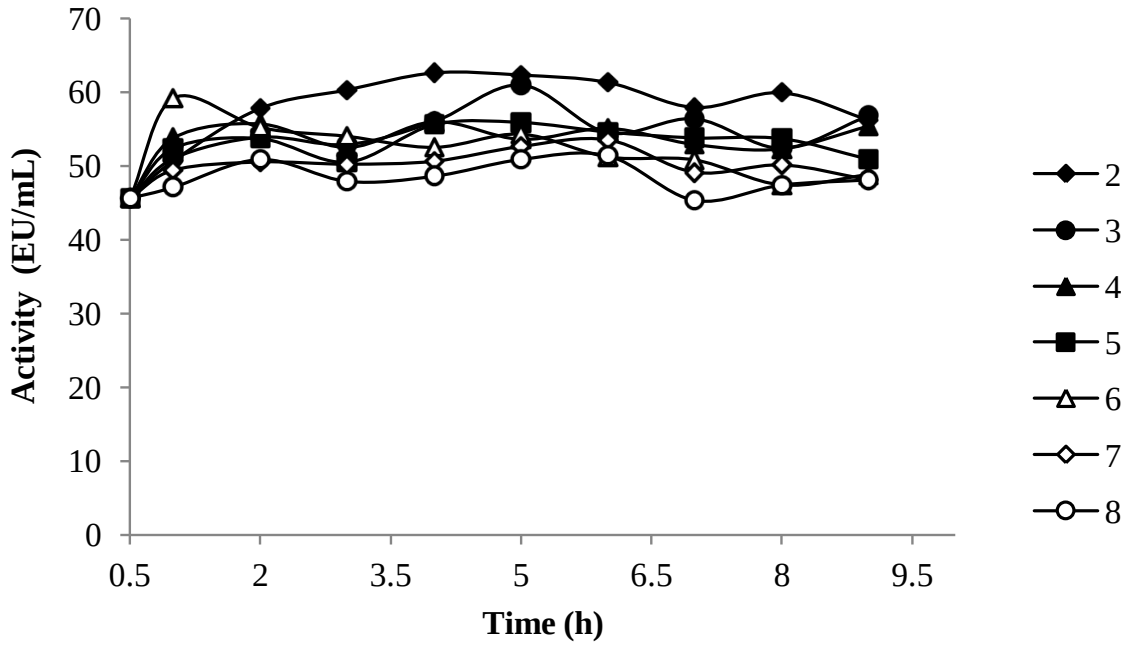

Figure 2. Effect of pH on the activity of the purifed phytase from *L. plantarum*. Enzymes and substrate were dissolved either in 10 mM buffers of various pH. Other conditions were as given for the standard assay method

Determining protein content

Bradford method was used to determine the amount of protein in the enzyme solution quantitatively. In this method, negatively charged Coomassie Brilliant Blue G-250, that binds positive charge in the proteins in acidic medium, is used as a dye. The sensitivity of this method is 1 to 100 µg (Bradford 1976).

Determining the effects of certain metal ions on enzyme activity

Effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on enzyme activity were also investigated. For this purpose, effects at 0.5 and 1 mM concentrations were investigated for each metal ion (Nadaroglu and Tasgin, 2013).

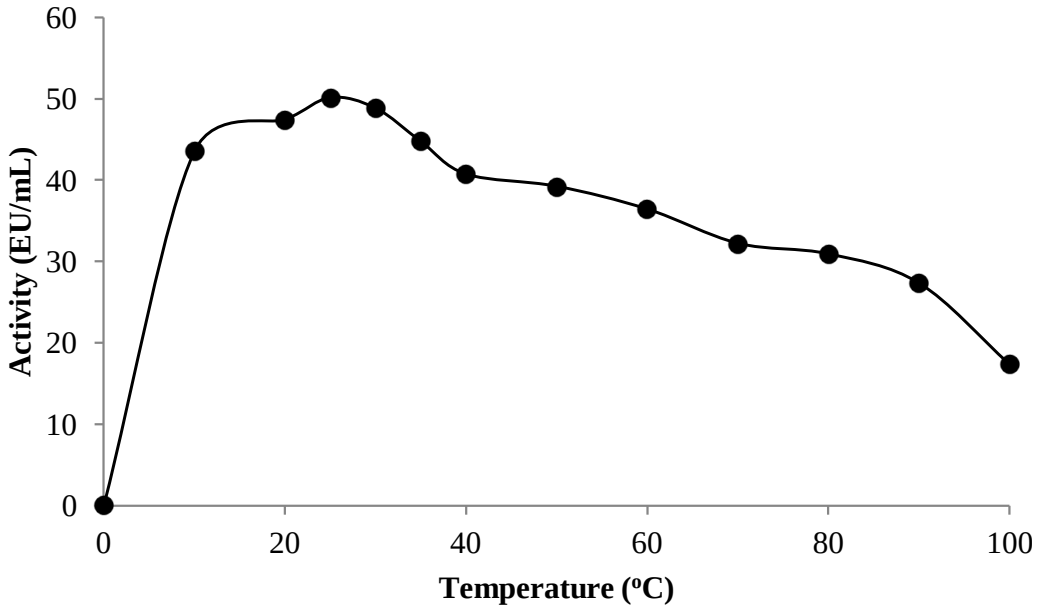

Figure 3. Effect of temperatures on the activity of purifed phytase from *L. plantarum.*

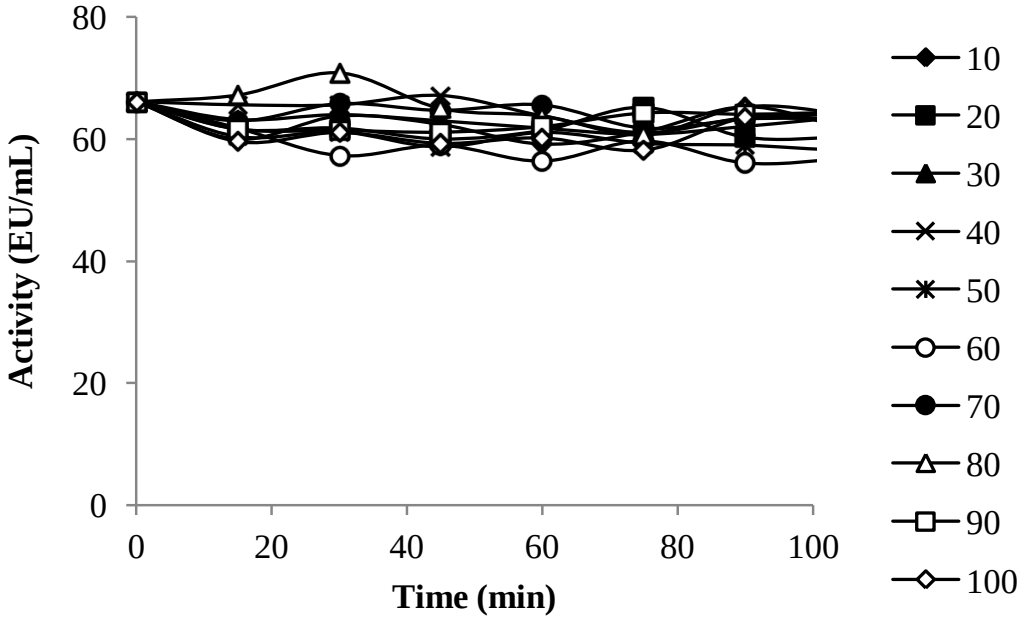

Figure 4. Effect of temperatures on the stability of the purifed phytase from *Lactobacillus plantarum*. Enzymes were incubated at different temperatures for various periods, then directly put into an ice water bath and the residual phytase activity was measured as described in the standard assay method.

RESULTS AND DISCUSSION

In this study, phytase enzyme activity and some kinetic properties of *L. plantarum*, which is a LAB used in many areas, were studied.

Extracellular enzyme activity of *L. plantarum* was determined as 167.3 EU/mL as a result of standard activity assay measurements (Table 1). Looking at the studies in the literature, Songre-Ouattara et al. (2008) have determined intracellular enzyme activity of *L. plantarum* 4.4 as 348.7±17.4 U/mL, intracellular enzyme activity of *L. plantarum* 6.1 as 276.3±51.4 U/mL, and

Table 1. The purification process of phytase from *Lactobacillus plantarum*.

Parameter	Volume (mL)	Activity (EU/mL)	Total activity EU	%	Protein amount (mg/mL)	Specific activity (EU/mg)	Purification fold
Crude extract	50	167.3	8365	100	6.59	25.39	-
Amonium sulphate precipitation (60-80%)	50	45.6	2280	27.3	0.29	157.75	6.21

Table 2. The effect of some chemical compounds on phytase activity

Chemical compound	Concentration (mM)	Relative activity (%)	Concentration (mM)	Relative activity (%)
None	-	100 ± 0.0	-	100 ± 0.0
Ca^{2+}	0.5	82.9	1	62.0
Fe^{2+}	0.5	153.8	1	235.6
Zn^{2+}	0.5	65.6	1	28.2
Cu^{2+}	0.5	65.6	1	8.16
Mn^{2+}	0.5	80.7	1	56.3

intracellular enzyme activity of *L. fermentum* 7.4 as 276.3±13.2U/mL. Our results comply with literature.

Phytase enzyme was partially purified by ammonium sulfate precipitation of the crude extract. For this purpose, ammonium sulphate precipitation was performed at 0-20, 20-40, 40-60 and 60-80% saturations in order to determine the precipitation range of phytase enzyme. It was determined that enzyme is precipitated in 80-100% range. The enzyme was 6.21-fold purified by this precipitation from *L. plantarum* a by 27.3 yield.

The optimum pH of the phytase enzyme purified from *L. plantarum* was 6 (Figure 1). It was observed that purified phytase enzyme is stable at pH 2.0-11.0 values for 10 h and the enzyme activity was not affected by change in pH much (Figure 2).

Haros et al. (2008) reported that the optimum activity of phytase isolated from *L. plantarum* W42 and *L. plantarum* JBRS is at pH 6.0-6.5, and the optimum activity of phytase isolated from *L. plantarum* 110 is at pH 7.5; and these are in line with our findings.

The activity was measured in the range of 10-100°C, in order to determine optimum temperature of the phytase enzyme obtained from *L. plantarum* (Figure 3). According to the results, the optimum temperature of the phytase enzyme from *L. plantarum* was 30°C. Pure phytase enzyme was incubated for 2 h at 10-100°C, and its temperature stability was found by activity measurements at certain intervals. For phytase enzyme, it was observed after 2 h that the enzyme was stable at the applied temperature values and maintains its 90% of activity (Figure 4).

Effects of certain metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} on the activity of phytase enzyme purified from *L. plantarum* were also studied. It was determined that Cu^{2+}, Mn^{2+} and Ca^{2+} inhibited pure phytase enzyme activity, whereas Fe^{2+} activates the enzyme (Table 2).

Shimizu (1992) found that the activity of extracellular phytase enzyme purified from *B. subitilis* (natto) N-77 strains is inhibited by addition of EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+}. Yoon et al. (1996) have found that *Enterobacter* spp., which is an extracelluar phytase producer, was inhibited by the addition of 4.1 mM Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and ethylenediaminetetraacetic acid (EDTA). Yanke et al. (1999) have determined that 5 mM concentration of Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} inhibits phytase enzyme of *Selenomonas ruminantum*, whereas Pb^{2+} activates the enzyme.

In a study conducted by Choi et al. (2001), it was found that the activity of extracellular phytase enzyme obtained from *Bacillus* spp. KHU-10 is inhibited by EDTA and metal ions such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, $Hg^{2+}$and Mn^{2+}. These literature findings support the findings in our study.

Conclusion

Based on the results of our study, it was revealed that the phytase enzyme purified partially from *L. plantarum* (ATTC) bacteria is extremely resistant against pH and temperature changes and metal ions that can be found in certain media; and maintains its activity for a long time. It was concluded that it would be appropriate to use the resulting phytase enzyme especially in food, feed and paper industries, in environmental treatment and in soil improvement by enabling its conversion to myo inositol derivatives through hydrolysis of phytic acid found in

grains and legumes.

Conflict of Interests

The author(s) have not declared any conflict of interests

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Full Length Research Paper

Comparitive studies on antibacterial activity of Patchouli [*Pogostemon cablin* (Blanco) Benth] and Geranium (*Pelargonium graveolens*) aromatic medicinal plants

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Solvent (Hexane, Ethanol, Methanol) extracts of Patchouli (*Pogostemon cablin*) and Geranium *(Pelargonium graveolens*) were compared for their potential antibacterial activity against four bacterial species using disc diffusion assayed method. DMSO and Chloramphenicol were used as negative and positive controls respectively. The growth inhibitory effect of the various solvents on *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Enterobacter aerogenes* were obtained and the most effective extract was the hexane extract of Patchouli compared to that of Geranium, which showed a maximum zone of inhibition (18 to 21 mm) against *S. aureus*. Ethanol and methanol extracts of Geranium showed maximum zone of inhibition (10 to 11 mm) against *S. aureus*. There was no zone of inhibition for aqueous, ethanol and methanol extracts of patchouli with 20 to 80 µl concentration and no zone of inhibition for aqueous, hexane extracts of Geranium with 20 to 80 µl concentration. The minimum inhibitory concentration (MIC) ranged from 40 to 80 µl for Patchouli hexane extract and MIC ranged from 60 to 80 µl for Geranium ethanol and methanol extracts. The potency of these extracts based on the zones of inhibition and MIC values were higher indicating that leaves have a potential broad spectrum antibacterial activity. The combination of these antimicrobial plant extracts can be used to treat infectious diseases in the near future.

Key words: Patchouli, geranium, plant extracts, disc diffusion, bacterial species, MIC, zone of inhibition, infectious diseases.

INTRODUCTION

Medicinal plants are the greatest economic source of the world. Nature has bestowed on us a very rich botanical resources and a large number of various types of plants grow in several parts of the world (Bishnu et al., 2011).

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Plants are the richest resource of drugs of modern medicines, traditional systems of medicine, food supplements, nutraceuticals, pharmaceutical intermediates, folk medicines, and chemical entities for synthetic drugs (Hammer et al., 1999; Bishnu et al., 2011). Herbal medicine is still the basis of about 75 to 80% of the whole population, and the plant extracts and their active constituents are used in the major part of traditional therapy (Akerele, 1993). Indigenous medicinal systems like Ayurveda, Unani and Siddha are used around 1500 plants systematically (Bishnu et al., 2011). Plant originated antimicrobials have more therapeutic potential (Salau and Odeleye, 2007; Girish and Satish, 2008). Lot of interest has been shown towards the exploitation of natural compounds for their antimicrobial activity over the past 2 decades (Werner et al., 1999; Samy and Ignacimuthu, 2000; Girish and Satish, 2008). Earlier findings have reported the efficacy of various herbal extracts against microorganisms confirming the fact that plants are the bedrocks for identifying novel antimicrobial molecules (Evans et al., 2002; Girish and Satish, 2008). The increasing interest on traditional ethno medicine may lead to discovery of novel therapeutic agents.

Human infections caused by microorganisms and fungi are rampant in the tropical and subtropical areas of the world (Girish and Satish, 2008). Traditional therapy involves the use of plant extracts or their active principles which may serve as source for modern drugs and intermediate compounds for synthesizing analog drugs with better desirable properties (Jones, 1996; Hammer et al., 1999). The aim of this work was to prepare aqueous organic (solvent) extracts of Patchouli (*Pogostemon cablin* (Blanco) Benth) and Geranium (*Pelargonium graveolens*) and compare their antibacterial activities with selected microorganism strains.

Patchouli (*P. cablin*) is a herb belonging to the Labiatae family originating from Southeast Asia. Patchouli leaves contain an essential oil which is made up of patchouli alcohol (patchoulol) as a major component and several other minor components such as caryophyllene, α-, β-, γ- and δ--patchoulene, pogostol, seychellene, cycloseychellene, α- and β-bulnessene, α- and δ-guaiene and norpatchoulenol (Akhila and Nigam, 1984; Akhila et al., 1988). The other species (*Pogostemon horthensis, Pogostemon hyneanus and Pogostemon plectranthoide*) of genus Pogostemon also yield patchouli oil in a lesser amounts than *P. cablin* (Ngampong et al., 2009).

P. graveolens (L.) belongs to the family Geraniaceae also called as Geranium is an erect, much branched shrub, which can reach a height of up to 1.3 m and a spread of 1 m. The essential oil produced from this plant has been used in the treatment of hemorrhoids, dysentery, heavy menstrual flows, inflammation and cancer (Ben et al., 2013). The French medicinal community currently treats diabetes, diarrhoea, gall bladder problems, gastric ulcers, jaundice, liver problems, sterility and urinary stones with this oil ((Peterson et al., 2006). The essential oil fraction of *P. graveolens* and its main components, geraniol and citronellol, exhibited additive effects with amphotericin B and with ketoconazole against both *Aspergillus* species, resulting in fractional inhibitory concentration (FIC) indices ranging from 0.52 to 1.00 (Shin, 2003).

MATERIALS AND METHODS

Collection of medicinal plants for the study

Patchouli (*Pogostemon cablin* (Blanco) Benth) and Geranium (*Pelargonium graveolen)* producing aromatic oil of medicinal importance were selected based on ethanomedical importance. Healthy and disease free leaves of *P. cablin* (Blanco) Benth collected from University of Agriculture, Bengaluru and Authenticated (Authentication No. 11) by Dr. Vasundhara, Professor, GKVK, Bengaluru. Geranium (*P. graveolens)* plants were collected in and around Hyderabad (CIMAP), Andhra Pradesh (India) and authenticated from Department of Botany, Osmania University (Voucher No. 094), Hyderabad, India. These leaves were used for the preparation of solvent extracts.

Test microorganisms

Authentic pure cultures of human pathogenic Gram positive bacteria like (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli, Enterobacter aerogenes*) were obtained from Department of Microbiology, Osmania University, Hyderabad, India. They were pre-cultured in nutrient broth culture overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min and pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically (A_{610} nm) (Suseem and Mary, 2012).

Preparation of extracts

Grinding of the selected plant materials

After drying under shade at 37°C for 7 days the plant materials were ground. To prevent the loss of active components exposure to direct sunlight was avoided as suggested by Girish and Satish (2008).

Aqueous extraction

50 g of dry powdered *P. cablin* and *P. graveolens* leaves were separately infused in distilled water, and the mixture was heated for 15 min at slow heat (Jigna et al., 2005; Soniya et al., 2013). The extract was then filtered using gauze and Whatman filter paper No.1, followed by sterilization via filtration through sterile syringe filter (0.2 -0.45 µm pore). The filtered extract was stored as aliquots for future use (Haitham et al., 2009).

Preparation of leaf solvent extract

Soxhlet extraction

The finely ground sample (100 g) of *P. cablin* and *P. graveolen* leaves was successively extracted with hexane, ethanol and methanol using the Soxhlet apparatus for 48 h each. The solvent extracts were concentrated under reduced pressure separately.

Table 1. Patchouli extracts concentration (40 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Patchouli extract concentration (40 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	16	--	--	--	24
Bacillus subtilis	--	12	--	--	--	19
Staphylococcus aureus	--	18	--	--	--	18
Enterobacter aerogenes	--	10	--	--	--	25

Table 2. Patchouli extracts concentration (60 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Patchouli extract concentration (60 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	20	--	--	--	24
Bacillus subtilis	--	19.3	--	--	--	19
Staphylococcus aureus	--	20	--	--	--	18
Enterobacter aerogenes	--	15.6	--	--	--	25

The mixture was filtered and dried using a rotary evaporator (Abah and Egwari, 2011). The dried materials were stored in sterile labeled bottles and kept as aliquots until further use.

Antibacterial assay

Leaf extracts (aqueous and organic) of Patchouli (*Pogostemon cablin* (Blanco) Benth) and Geranium (*Pelargonium graveolens)* were then tested for their antibacterial activity. The preparations of leaf extracts for antimicrobial activity were done by slight modifications to Alade and Irobi protocols (Kedarnath et al., 2012).

Disc diffusion method

Disc diffusion method was employed for the determination of antibacterial activities of the *P. cablin* and *P. graveolens* leaf extracts (NCCLS, 1997; Haitham et al., 2009). Nutrient agar and the petriplates were sterilized by autoclaving under aseptic conditions. 20ml of the agar medium was dispensed into petriplates in sterile conditions in a laminar flow to obtain plates of a uniform depth of 4mm. The overnight inoculums containing 106 bacterial cells/ml was spread on the surface of the solidified nutrient agar plates.

Whatmann No.1 filter paper was cut into small discs of diameter 6 mm and autoclaved (Kensa and Yasmin, 2011). Filter paper discs were impregnated into each plant extracts of different concentrations namely 20, 40, 60, 80 µl and dried aseptically. Using a sterile forceps, the treated filter papers containing *P. cablin* and *P. graveolen* leaf extracts were laid down on the surface of inoculated agar plate. in In addition a positive control disc (Chloramphenicol) and negative control disc (10% DMSO) were also added alongside the extract treated discs. All the plates were maintained as triplicates. The plates were incubated at 37°C for 24 h and the diameter of the zone of inhibition was measured in millimeter.

RESULTS

No positive results were found at a concentration of 20 µl in both the leaf extracts. The results of the antibacterial activity of Patchouli and Geranium aqueous and organic (Hexane, Ethanol, Methanol) extracts, assayed in vitro by the disc diffusion method are described in the tables that follow. The highest zone of inhibition and the effectiveness is the major consideration in the case of antibacterial activity. The growth inhibitory effect of *E. coli, B. subtilis, S. aureus* and *E. aerogenes* are presented in Tables 1 to 6 and Figure 1 to 2. In our study, the most effective activity was proven by Patchouli hexane extract in comparison to Geranium with a maximum zone of inhibition ranging from 16 to 20.5 mm against *E. coli*, 12 to 19.3 mm against *B. subtilis*, 18 to 21 mm against *S. aureus*, 10 to 15.8 mm against *E. aerogenes*. All other 3 extracts were ineffective. Ethanolic and methanolic extracts of Geranium showed effective activity against *S. aureus* than *E. coli, B. subtili* and *E. aerogenes* with maximum zone of inhibition ranging from 08 to 8.5 mm against *E. coli*, 10 to 10.5 mm against *B. subtilis*, 10 to 11 mm against *S. aureus*, 08 to 09 mm against *E. aerogenes* ethanolic extract and 8.5 to 9 mm against *E. coli*, 10 to 11 mm against *B. subtilis*, 11 to 11.5 mm against *S. aureus*, 9 to 9.5 mm against *E. aerogenes* with methanolic extract, respectively. The aqueous and hexane extracts of Geranium were not effective in inhibiting *E. coli, B. subtilis, S. aureus* and *E. aerogenes*. Geranium showed very low activity than Patchouli. The minimum inhibition concentration (MIC) of patchouli hexane extract ranged from 40 to 80 µl. In case of

Table 3. Patchouli extracts concentration (80 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Patchouli extract concentration (80 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	20.5	--	--	--	24
Bacillus subtilis	--	19.3	--	--	--	19
Staphylococcus aureus	--	21	--	--	--	18
Enterobacter aerogenes	--	15.8	--	--	--	25

Table 4. Geranium extracts concentration (40 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Geranium extract concentration (40 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	--	--	--	--	24
Bacillus subtilis	--	--	--	--	--	19
Staphylococcus aureus	--	--	--	--	--	18
Enterobacter aerogenes	--	--	--	--	--	25

Table 5. Geranium extracts concentration (60 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Geranium extract concentration (60 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	--	08	8.5	--	24
Bacillus subtilis	--	--	10	10	--	19
Staphylococcus aureus	--	--	10	11	--	18
Enterobacter aerogenes	--	--	08	09	--	25

Table 6. Geranium extracts concentration (80 µl) and their zone of inhibitions.

Test organisms	Zone of inhibition in diameter (mm)					
	Geranium extract concentration (80 µl)					Standard reference
	Aqueous	Hexane	Ethanol	Methanol	DMSO	Chloramphenicol
Escherichia coli	--	--	8.5	09	--	24
Bacillus subtilis	--	--	10.5	11	--	19
Staphylococcus aureus	--	--	11	11.5	--	18
Enterobacter aerogenes	--	--	09	9.5	--	25

Geranium's ethanolic and methanolic extract; the MIC ranged from 60 to 80 µl. These results indicate that leaves have a potential broad spectrum antibacterial activity (Table 1a to 1c; Table 2a to 2c).

In future, these extracts can be combined as a formulation to treat infections caused by the test organisms.

DISCUSSION

The present study revealed the antibacterial potential of Patchouli [*P. cablin* (Blanco) Benth] and Geranium (*P. graveolen*). Extracts of these plants hold active constituents with antimicrobial properties and appear to be potential antimicrobial therapeutic agents against

Figure 1. Patchouli (*Pogostemon cablin*).

Figure 2. Geranium (*P*elargonium *graveolens*).

infections caused by the tested pathogens in this study. Good results were achieved with nutrient agar showing visible zone formation indicating bacterial growth inhibition. Similar approach was used by other investigators but used different culturing media for growth of organisms (Senthilkumar et al., 2010). Use of different solvents helps us to isolate extracts containing higher active compounds from the plants. Many studies suggested that different solvent extracts of various plants has tremendous biological activity (Senthilkumar et al., 2010). Such an effective extract can be subjected to isolation of the therapeutic compounds and antimicrobials agents for further Pharmacological studies (Parekh and Chanda, 2006). Ethnobotanical approach is one of the universal practices applied in choosing the plants for pharmacological study (Cox and Balick, 1994) although, these plants declared the antibacterial activity against 4 medically important human pathogens. Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) are the matters under study. This study can be further extended to study other major pathogenic bacteria and develop a novel broad spectrum antibacterial formulation in future. Now, our research will be focused to develop a broad spectrum antibacterial combined herbal formulation with these plants.

Conclusion

The present study confirmed the antimicrobial properties of aqueous and organic (hexane, ethanol and methanol) extracts from Patchouli [*P. cablin* (Blanco) Benth] and Geranium (*P. graveolen)* that showed significant growth inhibition for *E. coli, B. subtilis, S. aureus and E. aerogenes*.

All these microorganisms pose serious threat to mankind because of their ability to produce resistant strains towards a spectrum of antibiotics thereby making them difficult to treat. The encouraging results obtained by us indicate the antimicrobial activity of *P. cablin* and *P. graveolens* which can be exploited as a natural antibiotic for the treatment of several infections caused by these organisms, and could be useful in understanding the relations between traditional cures and current medicines.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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