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African Journal of Agricultural Research

Table of Contents: Volume 11 Number 18, 5 May, 2016

ARTICLES

African Journal of Agricultural Research

Table of Contents: Volume 11 Number 18, 5 May, 2016

ARTICLES

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Review

Anatomical differences among forage with respect to nutrient availability for ruminants in the tropics: A review

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Different types of forage have been used worldwide in farm animal feeding. However, different microscopic cellular arrangements are clear in the anatomical structure, are due to climatic influences and are reflected in the productivity responses of cattle, especially in comparisons among tropical and temperate grasses. The physical impediment of the plant anatomical structure affects nutrient accessibility to rumen microorganisms. Thus, the objective of the present review was to discuss aspects related to C_3 and C_4 forage plant anatomy and nutrient availability for ruminants, due to the specific structure of each plant tissue, the speed at which the microorganisms can access the cells becomes different animal responses.

Key words: Plant anatomy, digestibility, nutritional value, neutral detergent fiber.

INTRODUCTION

Understanding the anatomical structure of forage can effectively influence nutritional aspects in ruminants. Both grass and legumes are angiosperms. However, their carbon fixation physiology and metabolism can differ among species. In most plants, the CO_2 fixation process includes a 3-carbon molecule as the first stable compound; this process is the C_3 Calvin-Benson cycle (Sharkey and Weise, 2015; Yamaoka et al., 2015). However, in certain plants, the first stable compound is a 4-carbon molecule in a process referred to as the C_4 dicarboxylic acid cycle (Gowik and Westhoff, 2011). The above-described changes imply different morphological traits. Furthermore, C_4 plants are more adapted to light and high temperatures (Valente et al., 2011a). In comparison, C_4 plants require less than 400 g water to produce 1 g dry matter (DM), whereas C_3 plants use 400 g to 1000 g water to produce the same quantity of DM (Odum, 1983). In nature, including forests, C_3 plants

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produce the most photo-assimilates in the world, likely because these plants are more competitive in mixed communities under the effects of shadow casting and little variation in temperature and luminosity (Jardine et al., 2014). When grass is intercropped with legumes in cattle-feeding pastures, high results of degradability are founded (Barcellos et al., 2008; Silva et al., 2010; Zhang et al., 2015). An advantage of intercropping is that legumes exhibit less variation in their nutritional value throughout the year, whereas the grass forage nutrient content decreased in dry seasons (Monteiro et al., 1998; Sage et al., 2015). Intercropping grass and legume forages produces imbalance problems in the pasture because all tropical legumes are C_3 plants, whereas most tropical grasses feature a C_4-type metabolism. In these mixed pastures, C_4 grass exhibits higher growth, and depending on animal management, legumes may disappear due to low persistence and grazing intensity (Carvalho and Pires, 2008a). Tropical C_4 plants exhibit little photorespiration because the high CO_2 levels in the bundle sheath cells accelerate the carboxylase reaction relative to the oxygenase reaction. This effect is relevant at higher temperatures. Thus, C_4 plants have an advantage in hot and bright environments (Van Soest, 1994; Sage and Stata, 2015).

Shaded plants invest relatively higher proportion of photoassimilates in increased leaf area, to maximize capture of the available light. Usually have thin leaves, higher specific leaf area and sheets with lower bulk density. The anatomical changes that occur on leaves develop under low light play an important role in plant adaptation to the conditions imposed by the environment. Generally these changes are related to the increased uptake and utilization of the incident light, feature that limits growth in the shade, increasing photosynthetic efficiency of the plant (Lambers et al., 1998). The increase of leaf area in low light conditions It is directly related to anatomical changes that may occur in the shaded plants as cuticles and thinner epidermis, mesophyll and thinner smaller proportion of palisade parenchyma tissue conductive support and a higher proportion of spaces intercellular and lower stomatal density (Berlyn and Cho, 2000).

In studied with response to levels of artificial shades (0, 50 and 70%) with the objective of determining the acclimation of C_3 and C_4 species of forage species to changes in the luminous environment, Gobbi et al. (2011) worked with C_4 plant signal grass (*Brachiaria decumbens* cv. Basilisk) and C_3 forage peanut (*Arachis pintoi* cv. Amarillo) and concluded that the specific leaf blade of the two species increased linearly as a function of the increasing levels of shading. In signal grass, the increase on specific leaf area was followed by a linear reduction in leaf thickness, with the increasing levels of shade. However, forage peanut leaf thickness was not significantly altered by shade. Stomatal density on adaxial and abaxial leaf surfaces decreased with the increase on levels of shade. The forage species evaluated showed a good acclimation to variations on light intensities, and they are good alternatives to use in environments with low solar radiation levels.

A great diversity of species of microorganisms present in the rumen environmental, with specific functions, in the degradation of carbohydrates, protein and lipids. However, the knowledge of the interactions between the different species of microorganisms in the rumen ecosystem. Our understanding of characteristics of the ruminal microbial population has opened new avenues of microbial ecology and the use of these nutrients in the plants (Krause et al., 2014; Valente et al., 2016). Thus, the study proposal was to discuss aspects related to C_3 and C_4 forage plant anatomy and nutrient availability for ruminants, due to the specific structure of each plant tissue, the speed at which the microorganisms can access the cells becomes different animal responses.

FORAGE PLANT STRUCTURE

Forage plants are composed of different structures, and the physical and chemical composition of each tissue is directly related to its structure in the plant. Supporting tissues must be densely grouped with thickened and lignified cell walls (Lempp et al., 2000). In photosynthesis-specialized tissues, the cell walls must be thin and non-lignified. Thus, the digestibility potential for given forage is related to the different tissues it comprises (Akin and Robinson, 1982; Batistoti et al., 2012; Cardoso, 2013). Accordingly, higher lignified vascular and sclerenchyma tissue content in a plant lowers the digestibility ratio (Brito et al., 2004; Queiroz et al., 2000b; Valente et al., 2011b,c). As the plant ages, the sclerenchyma cell walls in the leaf blades tend to thicken. In a study on three cultivars of elephant grass, which is tropical forage, Brito and Deschamps (2001) found that the lignified tissue area increased with plant growth in both the leaves and stem. The maturity stage is an important factor that influences the nutritional value of the forage plant (Wilson, 1997, Dabo et al. 1997; Aoki et al., 2013). In grass plants, leaf blades harbor different tissues, including tissues specialized in liquid conduction, tissues specialized in supporting the plant and tissues composed of mesophyll, where photoassimilate synthesis occurs. Conducting tissues are known as vascular bundles and are composed of xylem and phloem. Sclerenchyma is a supporting tissue. However, in grass, supporting and conducting tissues are associated. The epidermis covers the lower and upper outer surface of the plant and can feature a cuticle on its external surface. In grass, the stem is composed of an outermost epidermis and parenchyma tissue with vascular bundles that are scattered as a sub-epidermal sclerenchyma ring surrounding the entire stem (Wilson, 1997). The vascular bundles are similar to the bundles in leaves and can include a fiber ring (sclerenchyma) around each bundle. In the initial development stages, only xylem is lignified. However, as age increases, the maturation progress proceeds to lignify the sclerenchyma ring and, at a more advanced stage, even the parenchyma where the vascular bundles are inserted (Paciullo, 2000; Smith et al., 2013).

C_4-type forage plant species are anatomically different compared with C_3 plants. Specifically, the mesophyll cells are more densely arranged and form a radial structure around the vascular bundles, which is referred to as Kranz anatomy (Valente et al., 2011a) and is absent in C_3 plants. In a study comparing tropical C_4 and temperate C_3 grass tissue contents, Wilson (1997) showed that the higher mesophyll (rapidly degradable tissue) content in temperate grass produces a better cattle weight gain response than tropical grasses.

PLANT TISSUE CONTENT AND DIGESTIBILITY

Forage plant digestibility depends on its bromatological and histological composition. Although lignin is the major digestibility-limiting factor (Jung and Deetz, 1993), other components can also limit a plant's nutritional value (Bonelli et al., 2013). According to Van Soest (1994), these components include substances with different effects, such as those designed for plant defense through diminishing the forage palatability. Other substances inhibit animal metabolism by hindering bacterial development in the rumen; these include phenylpropanoids, such as lignin, flavonoids, isoflavonoids, other compounds with alkaloid and terpene content and tannins. However, tannins are classified into hydrolysable tannins (HT), with molecular weight between 500 and 3.000 g mol^{-1}, and condensed tannins (CT) with molecular weight up to 20.000 g mol^{-1}, second Cieslak et al. (2013). Tannins can bind proteins, and to a lesser extent metal ions, amino acids, and carbohydrates in aqueous solution (Makkar, 2003). Tannins can antimicrobial effects due to their capacity to bind microbial enzymes or cell wall or membrane proteins, or proteins in the substrate, decreasing microbial attachment and digestion (Morales and Ungerfeld, 2015).

Tropical Legumes also have tannins higher (e.g. *Desmodium, Cajanus*) or less degree (*Arachis, Neonotonia, Centrosema*), which interfere in palatability (decreased) in risk of bloat (reduces), digestion and utilization of protein and forage carbohydrates. However, depending on the nature and concentration of these tannins in forage, some advantages can be obtained, especially at high protein diets (e.g. Leucaena grazed in water). If this protein is not was complexed in part, it is precariously utilized by the animal, since the high rate of degradation in the rumen was not synchronized with the power supply. Then, N losses occur, excreted as urea for example second Barcellos et al. (2008).

In tropical climates, forage typically features less soluble carbohydrate and high cell wall content. Thus, forage tends to exhibit less nutritional value and more structures that are protective against predation. Due to influence by environmental factors, such as long periods of hot nights, plants respire and, when forage grows at higher temperatures, lignification increases. Thus, forage with a C_4 metabolism is typically lower quality (Figueiras et al., 2015). Also, according to Van Soest (1994), the generalization that C_4 plants have a lower nutritive value than C_3 is not universally true, and a few exceptions deserve mention. Corn and sorghum are C_4 plants derived from tropical ancestors. Studies show that corn grown under tropical conditions has a much lower nutritive value than corn grown in a temperate environment. legumes usually have higher levels of crude protein as compared to tropical grasses, which can improve the protein balance in the rumen ecosystem.

Studies on leaf anatomy and its relationship with forage plant nutritional value have been reported (Wilkins, 1972; Wilson, 1976; Rossatto et al., 2015). For tissues that are digested after reaching the rumen, different types of microorganisms must colonize the food particles. The main invasion route seems to be via lacerated epidermal areas. Brito and Deschamps (2001) found that stomata in the epidermis of sheaths and blades were used by microorganisms to access inner tissues. The degradation process begins in the substomatal cavity and progresses through the mesophyll. Rumen bacteria initially digest mesophyll and phloem cells (Hanna et al., 1973; Akin et al., 1973; Akin, 1989). To obtain access to parenchyma bundle sheath cells, microorganisms must first digest the mesophyll or epidermal cells, or the bundle sheath cells must be exposed through physical damage. Thus, the parenchyma bundle sheath cell digestion rate is influenced by the mesophyll cell digestion rate, and the colonization time can decrease as the number of lacerated areas in the epidermis increases. Chewing by the animal can contribute to higher physical degradation of the grass, during both intake and rumination (Krause et al., 2014; Valente et al., 2016).

For the parenchyma bundle sheath and the epidermis, partial digestion is evident after 24 to 48 h of incubation (Bohn et al., 1988; Wilson et al., 1991; Wilson, 1993; Zhang et al., 2015). However, epidermal cells disappear completely after 24 h of incubation (Akin and Burdick, 1975; Sun et al., 2008). Second Moore and Jung (2001) lignification tends to be most intense in structural tissues such as xylem and sclerenchyma are virtually indigestible and remain intact in the ruminal fluid after long incubation times (Figure 1). The reason dry matter digestibility is negatively correlated with lignin concentration is because the concentration of lignin always increases as cell-wall concentration rises, and forage cell walls are always less digestible than cell soluble. There are large differences in lignification between grasses and legumes and also differences in the impact of lignin per se on their forage quality. Lignin concentration of legumes often appears

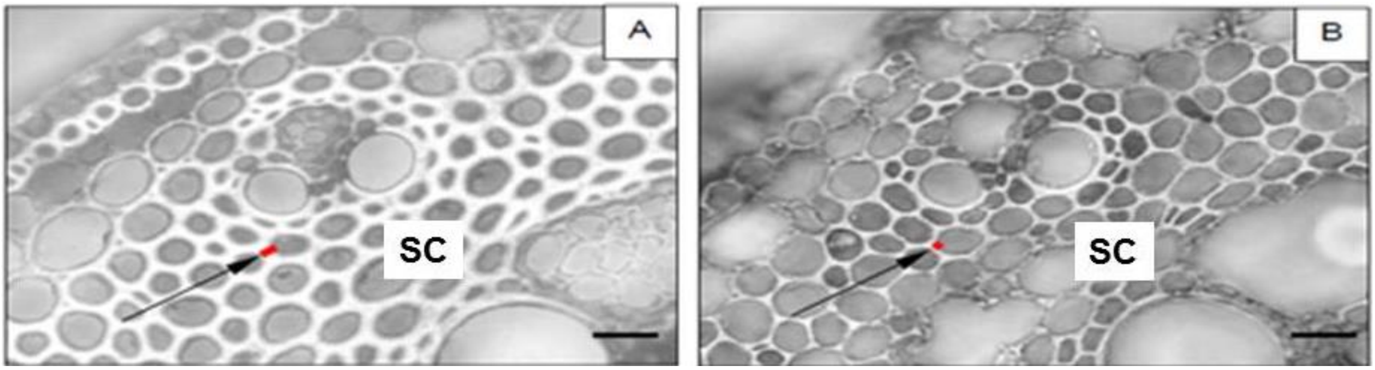

Figure 1. Brachiaria grass stem segment cross-section before (A) and after (B) 46 h of digestion. SC – sclerenchyma → cell wall. (— 15 µm) (Paciullo, 2000).

comparable to that of grasses when expressed as a proportion of dry matter. However, when expressed as a proportion of fiber, legumes demonstrate a wider range of lignin concentrations that are generally higher than those of grasses.

Inaccessibility of the secondary cell wall hinders microorganism entry and thus impedes rapid digestion of the plant cell wall, which is due not to its composition but to the time required to access the wall, which can then be excreted in the feces undigested (Wilson, 1997). Cell wall thickness can impede rapid access by the bacteria to initiate the digestive process. Digestion of the sclerenchyma cell wall, for example, requires 48 h. Thus, even if the cell wall is accessible to microorganisms, it will not be fully digested during its residence in the rumen (Krause et al., 2014). Thicker secondary walls require a longer time for full digestion (Akin and Robison, 1982; Monties, 1991). After compiling results from different works on tissue digestion, Akin (1989) suggested dividing C_4 grass leaf tissue into rapidly digestible*, e.g.*, mesophyll and phloem; slowly and partially digestible, *e.g.*, epidermis and vascular bundle sheath cells, respectively; and indigestible tissues, *e.g.*, xylem and sclerenchyma. In C_3 plants, only vascular tissue (except phloem) and the inner bundle sheath, which is observed in certain species, are resistant to digestion, and the sclerenchyma and parenchyma bundle sheath are considered slowly digestible. Tissues such as mesophyll and other types of parenchyma are not lignified, contain a large proportion of metabolites, and are highly degradable. Between these extremes are tissues with intermediate and variable degradability such as phloem and collenchyma (Wilson, 1993). Tissues such as the mesophyll, epidermis and outer bundle sheath are rapidly digested.

In general, C_4 species exhibit more vascular tissue, parenchyma bundle sheath and sclerenchyma content in the leaf blade, whereas C_3 plants feature exceptionally greater mesophyll content, which occupies approximately 60% of the cross-section of such grass (Minson and Wilson, 1980). The C_4 grass mesophyll is more slowly digested, even under rapid ruminal degradation, than C_3 species due to high cell density in C_4 forages. In C_3 species, cells are more loosely arranged and exhibit few cell-to-cell adhesion points. Anatomically, parenchyma sheath degradation seems the greatest cause of digestibility variations among C_4 photosynthetic *Panicum* spp. (Akin, 1982). Non-lignified tissues (collenchyma, chlorenchyma, cambium, phloem, and parenchyma) are more rapidly and almost completely degraded in animal digestive system, whilst lignified tissues, such as xylem vessels and phloem and xylem fibers, typically resist degradation. Epidermal and collenchyma cells develop very thick, but not lignified primary cell walls, which are slowly degradable, whilst waxy cuticle tends to resist degradation (Zorić et al., 2014).

Tissue degradation decreases with plant age. However, according to Carvalho and Pires (2008b), the environment also exerts an influence. Increases in lignified vascular tissue in elephant grass correlate with increasing tissue age, which is more evident in the stem (Brito and Deschamps, 2001). Second, Oliveira et al. (2014) with the aim to determine ruminal degradation of neutral detergent fiber of grasses of the genus *Cynodon* spp., harvested at four cutting ages. Concluded that daily increased in the cutting age, there was a linear reduction in the effective degradability of neutral detergent fiber (NDF) of blade and stem of 0.16 and 0.18%, respectively. The increase in the cutting age had a linear and positive influence on the un-degradable NDF with daily increments for leaf and stem of 0.12 and 0.18%, respectively.

Generally, mesophyll content is positively correlated with digestibility and negatively correlated with cell wall content. On the other hand, parenchyma bundle sheath, vascular tissue and sclerenchyma content are negatively correlated with digestibility and positively correlated with cell wall content (Queiroz et al., 2000a, b). Tropical grasses can exhibit different results for digestion of each leaf tissue. Mesophyll and phloem cells can be rapidly degraded after an incubation period of 12 to 24 h (Márquez et al., 2009), but these tissues have also been found undigested even after 48 h of incubation (Akin et al., 1983). In a study with Tifton 85 and Brachiaria grasses, Paciullo (2000) found that the mesophyll, parenchyma and phloem cells were the only fully digested cells *in vitro* upon up to 48 h of incubation. During his work with two *Cynodon* spp., Akin (1982) found that the mesophyll and phloem were the first tissues digested. *In situ*, after 24 h of digestion, only xylem and sclerenchyma were detected from temperate climate forage (Gasser et al., 2005). Compared with lignified tissues from palisade grass (*Urochloa brizantha* (Hochst. Ex A.Rich.) R.Webster) and creeping signal grass (*U. humidicola* (Rendle) Morrone & Zuloaga), Brito et al. (2004) found the highest content in palisade grass in the central basal and aerial apical regions.

Barcellos et al. (2008) in a compilation of data to assess the digestibility coefficient for legumes, found 64.4 for *Arachis pintoi,* 49.2 for *Stylosanthes Guianensis,* 44.3 for *Glycine wightii,* 55.6 for *Leucaena leucocephala* and 59.9 for *Medicago sativa.*

When Paciullo (2000) compared digestion among three tropical grasses, Tifton 85, Bermuda grass ((*Cynodon dactylon* (L.) Pers.) exhibited better results than molasses and signal grass species. As for molasses grass (*Melinis minutiflora* P. Beauv.), the high xylem and sclerenchyma content indicates that this species exhibits anatomical traits more typical of forage with lower nutritional value. signal grass species were intermediate compared with the two other species; it exhibited the highest cell wall thickness irrespective of developmental stage, which negatively affected digestion in the cross-sections. The sclerenchyma cell wall digestion rates ranged from 0.007 to 0.018 µm/h depending on the grass species and age and were higher with a 46 h incubation time. Thus, even without lignification, as in young stems, cell wall digestion in ruminal fluid was not complete during the incubation time, which suggests that limited digestion in thick-walled cells (greater than 1 µm) is mainly due to structural problems (Paciullo, 2000). According to Baurhoo et al. (2008), purified lignin can be digested by monogastric and ruminant animals under specific conditions. However, this phenolic polymer does not naturally occur in original plant structures (plant cell walls) and thus is not relevant to the present study.

Lignified vascular tissues exhibit highly significant positive correlations with NDF, acid detergent fibers (ADF) and lignin content and highly significant negative correlations with crude protein (CP) content (Gomes et al., 2011; Mokhele et al., 2012). These tissues exhibit thickened lignified cell walls, which are frequently associated with the slowly digesting fraction and forage fiber content (Queiroz et al., 2000b).

RELATIONSHIP BETWEEN VEGETAL ANATOMY AND CONCEPT THE FIBER IN RUMINANT NUTRITION

For determining forage quality is based on separating several fractions of the forage using detergents. A neutral detergent solution is used to dissolve easily digestible substances, which leaves a fibrous residue referred to as NDF that contains the main plant cell wall components (cellulose, hemicellulose and lignin) and generally corresponds to 600 to 800 g/kg forage DM. Thus, carbohydrates are the main energy source for ruminants both directly through absorbing their monomers in the digestive system and indirectly by conversion into volatile fatty acids through microbial fermentation (Aschenbach et al., 2014). The term fiber is used to define a nutritional rather than an anatomical concept. Fibrous carbohydrates and lignin, which compose the plant cell wall, are slowly digested, exhibit variable nutritional availability and occupy space in the gastrointestinal tract (Van Soest, 1967; Allen, 1996). Fiber methods isolate different chemical constituents in feeds. The fiber with the smallest magnitude is crude fiber, because the strong acid and alkali in this method leaves a residue that is mostly cellulose with variable amounts of lignin and hemicellulose. ADF is next largest in magnitude because it recovers most, if not all, of the polymeric lignin and cellulose in feeds, with some contamination from pectin, hemicellulose, tannin-protein complexes, and ash. Of the three routine fiber methods only NDF isolates all of the insoluble fiber components in plants (hemicellulose, cellulose, and lignin) with some protein contamination. In animal byproduct feeds, NDF isolates the nitrogenous material that is indigestible or slowly digesting and thus meets the requirements of the nutritional definition of fiber. Because ADF does not contain hemicellulose it is not an accurate estimate of fiber in feeds. It was developed as a preparatory step for the determination of lignin and was never intended to be a measure of fiber in feeds (Van Soest, 1994). The concept of physically effective fiber (peNDF) was introduced to account for the physical characteristics of NDF (primarily particle size) that affect chewing activity (saliva secretion) (Yang and Beauchemin (2007). This concept is based on the hypothesis that the fiber in long feed particles (>1 cm) promotes chewing and saliva secretion, which helps neutralize the acids produced during ruminal digestion of feeds. The fiber that promotes chewing is considered physically effective. The peNDF content of the diet can be determined by multiplying the NDF content of the diet by its physical effectiveness factor (pef) second Beauchemin and Yang (2005).

NDF AND INDF BEHAVIOR IN THE RUMEN

Of all routinely measured feed constituents, NDF is most consistently correlated with DM ingestion (Van Soest, 1994). Higher NDF intake occupies more space in the ruminant gastrointestinal tract, which produces a filling effect (Mertens, 2003). Furthermore, due to the lower rate of NDF disappearance in the digestive system, more time is required to reduce the particle size, which is necessary for escape from the rumen.

Forage fiber or plant cell wall content can be divided into a potentially digestible and indigestible fraction (pdNDF and iNDF, respectively). The digestible fraction of the fiber disappears from the rumen through digestion, fermentation and passage, whereas the indigestible fraction disappears solely through passage (Krause et al., 2014). Notably, particle density is affected by fermentation because the generated and retained gas tends to reduce the specific gravity. As fermentation proceeds and the concentration of potentially fermentable nutrients decreases, the gas production rate decreases, and the density increases, which allows for passage through the reticulo-omasal orifice (Allen, 1996). With the progression of forage maturity, iNDF content increases and decreases the microbial digestion rate of this fraction, which thus affects the fiber particle residence time in the rumen (Jung and Allen, 1995). Microbial growth in animals fed low-quality tropical forage is not yet fully understood. However, additional supplementation with nitrogenous compounds to improve animal performance yielded a balance between digestion and degradation; increased feed intake correlated with increased passage rate (Detmann et al., 2009). Studies on NDF digestibility, which is a heterogeneous fraction composed of two types of carbohydrates, hemicellulose and cellulose, and one phenylpropanoid, lignin, report varying content and physical structure in this fraction among plant species and during the plant life cycle (Gomes et al., 2011). Understanding the iNDF fraction is relevant because it plays an important role in ruminant nutrition. According to Valente et al. (2015), differences in feed particle size are reflected in the iNDF fraction, where a simple change in the particle size from 1 to 3 mm can dramatically increase the expected time for detecting the iNDF in the laboratory after ruminal incubation.

The disappearance of NDF from the ruminal environment is a time-dependent process that integrates the speeds of pdNDF degradation and iNDF escape from the ruminal environment (Ellis et al., 1994). Thus, the entry of new fibrous substrates from forage will only occur when part of the resident mass is removed from the environment through degradation or passage. Accordingly, the pdNDF yield can be maximized by minimizing the ruminal fill effect (Figueiras et al., 2010, 2015). This behavior indicates that removing iNDF from the ruminal system improves NDF intake from low-quality forage.

At an advanced maturity stage, forage exhibits lower degradation and fiber digestion rates, tends to increase ruminal fill and reduces the passage rate (Kp) (Van Soest, 1994). Passage rates can be mathematically calculated based on independent variables related to the animal or diet. In predicting the Kp potential, special attention must be paid to the ingestion of indigestible particles and to the animal's live weight (Cannas and Van Soest, 2000). Changes in the fibrous particle passage rate seem directly associated with a higher NDF degradation rate (Russell, 2002; Detmann et al., 2014). The pdNDF degradation reduces gas production and increases the relative concentration of the iNDF fraction of the particle, which is typically denser due to higher phenolic compound content.

CONCLUSION

C_4 grass mesophyll is more slowly digested, even under rapid ruminal degradation, than C_3 species due to high cell density in C_4 forages. In C_3 species, cells are more loosely arranged and exhibit few cell-to-cell adhesion points. Tropical forage typically features less soluble carbohydrate and high cell wall content. Lignification tends to be most intense in structural tissues such as xylem and sclerenchyma are virtually indigestible and remain intact in the ruminal fluid after long incubation times.

Plant cell wall lignification is a process that cannot be avoided because it would harm plant structure; however, it hinders nutrient availability for ruminants. Plants with higher growth rate have higher accumulation of lignin hindering access to components of the cell wall by microorganisms because increase the iNDF.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Resistance characterization of Italian ryegrass (*Lolium multiflorum*) biotypes to clethodim herbicide

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The objective of this study is to determine the dose required to control 50% population (C_{50}) and to reduce 50% dry matter production (GR_{50}) of resistant biotypes of ryegrass in comparison to that of a susceptible biotype as well as to evaluate the mechanism of resistance by cyt-P450 inhibitor application. The study was conducted in a greenhouse in Rio Grande do Sul, Brazil on plants that survived clethodim herbicide application, which were suspected of possessing resistance. For plants surviving field application, the biotypes were 50% controlled with herbicide dose of 28.4- and 29.5-times greater compared to that of susceptible biotypes; 50% of dry matter reduction occurred with doses of 540- and 574-times greater than the susceptibility dose of a biotype, since the dose required to reduce 50% of susceptible biotype was 0.2 g a.i. ha^{-1}. The biotypes showed metabolism of clethodim herbicide as regards the inhibition by piperonyl butoxide, indicating that metabolism is the probable cause of control failures in the field.

Key words: *Lolium multiflorum*, acetyl coenzyme A carboxilase, weed, mechanism of resistance; metabolization.

INTRODUCTION

Resistance is the inherent capacity of some biotypes to survive and reproduce after exposition to herbicide dose, which is otherwise lethal to the susceptible population of the same species (Christoffoleti and Lópes-Ovejero, 2008). The onset of resistance to a particular herbicide in a plant population occurs as a result of selection of pre-existing resistant biotypes under selection pressure exerted by repeated applications of the same active ingredient (Roman, 2007).

In the last growing season (2012) in Brazil, a reduction

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of efficiency of ryegrass control with ACCase-inhibiting herbicides was reported. Failure was observed in areas treated with herbicides aryloxyphenoxypropionates and cyclohexanediones on isolated plants (Fraga et al., 2013). Case analysis did not reveal any plausible explanation for the failure, since the application factors such as the herbicide dose, vegetative stage, environmental conditions, and equipment aids used were suitable. Furthermore, faults occurred in only one species present in the area, *Lolium multiflorum*, and applications with accurate spraying in greenhouse showed similar results.

The quantitative characterization of the resistance level can be performed by dose–response curves, which allow identification of the herbicide dose that provides 50% of population control (C_{50}) or 50% reduction in dry matter (DM) production (GR_{50}) (Burgos et al., 2013). With the knowledge about these variables, the resistance factor (RF) can be calculated, which refers to the number of times of dose application required for controlling the resistant population was greater than the dose that induced the same effect on a susceptible population (Hall et al., 1998).

Herbicide resistance can be endowed by target-site and/or non-target site-resistance. The target-site resistance can occur by gene point mutations resulting in amino acid changes in a target enzyme, which prevents or reduces herbicide binding (Powles and Yu, 2010). The non-target-site resistance can be conferred by enhanced activities of metabolic enzymes, reducing the amount of herbicide reaching the target-site below the lethal level and/or protecting plants from herbicide damage (Yu and Powles, 2014). Cytochrome P450 monooxygenase, glycosyl transferase (GT), glutathione S-transferase (GST) are the major super-families involved in herbicide metabolism (Yuan et al., 2007).

The cytochrome P450 inhibitors as piperonyl butoxide (PBO) can inhibit *in vivo* the metabolism of some herbicides, thus reversing resistance (Preston et al., 1996; Wang et al., 2013). We aimed to determine the parameters of C_{50} and GR_{50} of ryegrass clethodim resistant and susceptible biotypes and to investigate the response of these biotypes to clethodim application after treatment with the metabolic inhibitor of cyt-P450 monooxygenase.

MATERIALS AND METHODS

To achieve the first objective of this work, was conducted a trial of dose–response curve from August to November 2013 with ryegrass (*Lolium multiflorum*) plants from the commercial fields of the municipality of Coqueiros do Sul, Rio Grande do Sul (S 28°11′13″W 52°44′34″). Eight biotypes that survived 96 g a.i. of clethodim (Select 240 EC®) were collected and the trials were conducted in greenhouse at Federal University of Pelotas – Capão do Leão, Rio Grande do Sul. The collected plants had between 6 and 8 tillers, were separated after collection and originated plants (clones) were evaluated in subsequent experiments. The ryegrass plants that extracted with tillers were isolated for further study. The tillers were transplanted to pot soil and submitted to mass selection in a greenhouse with a dose of 108 g a.i. of clethodim. In this experiment, biotypes showing less phytotoxic effect of the herbicide were nominated as COQ 6 and COQ 7. In a second separation of tillers from the mother-plants, clones were obtained for the formation of experimental units for conducting the dose–response trial. Plants from the same collection sites of resistant biotypes were evaluated, but those that did not survive the herbicide application in the first selection were used to provide the susceptible plants.

The experimental design was a randomized block with four replications, and the experimental units were composed of 550-mL-pots, with one plant per pot. The treatments were arranged in a factorial design, where the factor A tested ryegrass biotypes susceptible (SUS) and resistant (COQ 6 and COQ 7), while the factor B was compared with the effect of increasing doses of the clethodim herbicide (Select 240EC®).

To determine the C_{50} and GR_{50} values, increasing doses of clethodim herbicide were applied (0, 13.5, 27, 54, 108, 216, 432, and 864 g a.i. ha^{-1}), considering 108 g a.i. ha^{-1} as the dose to control ryegrass. Treatments were applied post-emergence, when the clone plants were in their four-leaf stage. For this purpose, we used backpack precision sprayer pressurized with CO_2, equipped with nozzle-type fan 110.015 that distributed spray volume of 120 L ha^{-1}. The adjuvant Lanzar® was added to the water at a dosage of 0.5% (v/v).

The evaluated variables included control and DM of shoots of all biotypes. The control was visually assessed by two raters at 20 and 32 days after herbicide application (DAHA) on the percentage scale, where zero (0) represents no symptoms and hundred (100) represents plant death (Frans et al., 1986). At 32 DAHA, the plants were collected to determine the DM production. For this purpose, the dry plant material was subjected to stove treatment with forced air circulation in an oven at 60°C until constant weight is achieved. The dry weight was converted into percentage form and the materials obtained after treatments with herbicide were compared with the control.

The data were analyzed for normality (Shapiro–Wilk test) and then subjected to the analysis of variance ($p \leq 0.05$). For data found to be statistically significant, regression analysis was performed for the dose factor, and the biotype factor was preceded by comparing C_{50} or GR_{50} of resistant and susceptible biotypes.

Regression analysis was performed by using the SigmaPlot 10.0 program and adjusting the data to the sigmoidal regression equation of the logistic type, as follows:

$$y = a / [1 + (x / x_0)^b]$$

Where, y = percentage control; x = herbicide dose; e a, x_0 e b = equation parameters, a is the difference between the maximum and minimum points in the curve, x_0 is the dose that promotes 50% of response of variable, and b is curve declivity.

The values of C_{50} and GR_{50} were obtained by calculation of necessary value to promote 50% of control or dry matter reduction, according to parameters generated in the curves equations. From the values of C_{50} and GR_{50}, the RF for each biotype with suspected resistance was obtained by comparison with the results of the susceptible plants. For this was necessary to check the interval of confidence ($p \geq 0.95$) of susceptible biotype in relationship to resistant biotypes calculated by standards errors and standard

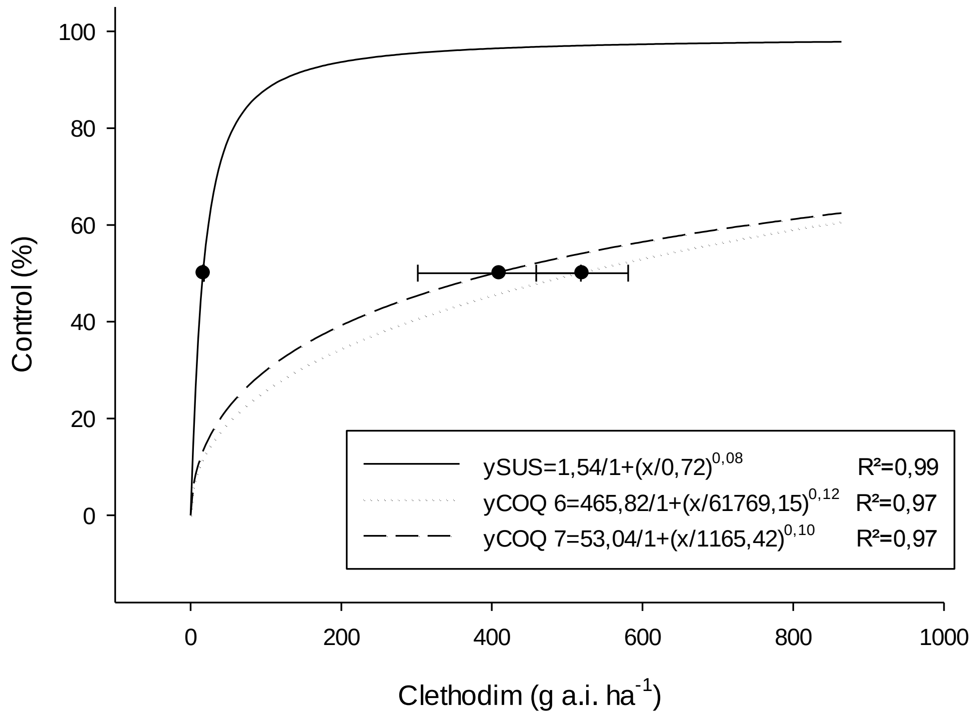

Figure 1. Control (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 20 days after herbicide application (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of plant control.

deviation from the curves equations. The overlap of interval of confidence of susceptible biotype in relation to resistant biotypes evaluated indicates that there was no significance difference between C_{50} and GR_{50} of biotypes (Avila et al., 2005).

For the realization of the trial of clethodim metabolism conducted from August to October 2014, plants that survived (COQ 8) clethodim application in the fields of Coqueiros do Sul city (same location as in the first experiment) were collected. Therefore, 120 g a.i. ha^{-1} of clethodim in was applied a 6-ha area by collecting the surviving plants at 30 DAHA. For result confirmation, repeated application was performed at the greenhouse, according to the methodology used in the first experiment.

The treatments were arranged in a factorial design with the factor A composed of ryegrass biotypes (COQ 8 and SUS) and factor B composed by application of cyt-P450 monooxygenase inhibitor Piperonyl Butoxide (PBO) alone and by preceding 30 min before the clethodim application, clethodim alone, and a no-treated control.

The plants were subjected to herbicide treatment when at the four-leaf stage with a tiller. At 30 min before application of clethodim, the PBO metabolism inhibitor was applied at a dose of 2100 g a.i. ha^{-1} (Preston et al., 1996) under the same mode as that in the dose-response trial.

The variables analyzed included control at 14 and 28 DAHA and DM of shoots at 28 DAHA, as for the first experiment. The data were analyzed for normality by the Shapiro-Wilk test, followed by analysis of variance ($p \leq 0.05$). For statistically significant results, the Tukey test ($p \leq 0.05$) was performed for biotypes factors and inhibitors.

RESULTS AND DISCUSSION

For the dose–response curve trial, an interaction was considered between the biotope factors and dose for all variables. While assessing the control at 20 DAHA, resistant biotypes were less affected at the same dose as for the susceptible plants (Figure 1). It was observed that, for 50% control of SUS, 17 g a.i. ha^{-1} clethodim was needed, and 520 and 410 g a.i. ha^{-1}, respectively, were needed for the resistant biotypes COQ 6 and COQ 7. The recommended dose of clethodim (108 g a.i. ha^{-1}) resulted in the control of 88% of the SUS and 32 and 35% for resistant biotypes COQ 6 and COQ 7, respectively.

Evaluation at 32 DAHA for the SUS revealed 50% control at a dose of 9.5 g a.i. ha^{-1} of clethodim. As for the resistant biotypes COQ 6 and COQ 7, the dose required was 270 and 280 g a.i. ha^{-1}, respectively, to achieve 50% control (Figure 2). The dose of 54 g a.i. ha^{-1} corresponding to half dose of clethodim to control ryegrass was sufficient to achieve >95% control in the susceptible biotype. This biotype was controlled at the maximum (100%) with of 108 g a.i. ha^{-1}. The resistant biotypes COQ 6 and COQ 7 were controlled by 68 and 66%, respectively, at the dose of 864 g a.i. ha^{-1}, 8 times

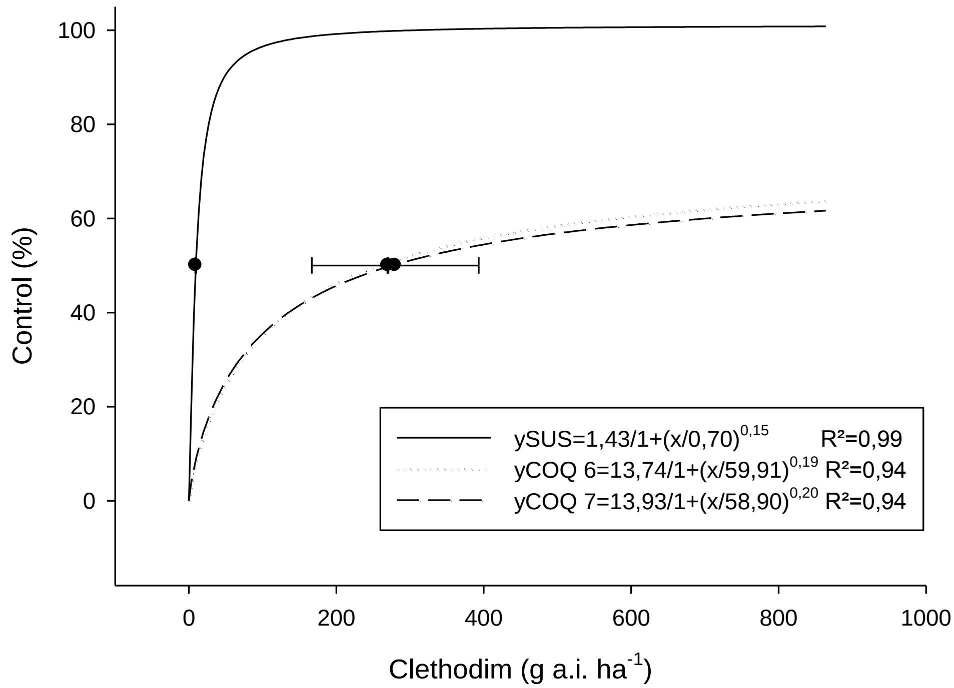

Figure 2. Control (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of plant control.

the registered and highest dose evaluated in this study.

The genetic variability of weed population is affected by several evolutionary factors such as the production system, interactions between culture and weed, gene flow, dispersal, and natural selection (Huangfu et al., 2009). Thus, the differences between the evaluated ryegrass biotypes may arise from intrinsic genetic characteristics of the biotypes that influence the response to herbicides. The genetic constitution can determine susceptibility or resistance to herbicides (Hartwig et al., 2008). However, considering that both the susceptible biotype as resistant biotypes share the same origin, it is possible that the differences observed between the evaluated biotypes occur due to cultural practices, especially by the herbicide action.

The RF at 20 DAHA was 30.5 and 24.11 for the resistant biotypes COQ 6 and COQ 7, respectively (Table 1). At 32 DAHA, last time of evaluation, the value of the RF for the biotypes COQ 6 and COQ 7 was 28.4 and 29.5, respectively (Tab. 1). These high RF values can be attributed to the times of plant collection to determine the shoot DM, since, at 32 DAHA, the resistant biotypes showed strong growth, while the SUS was controlled at 20 DAT.

The evaluation of the DM of shoot after 32 DAHA confirmed the control results observed, owing to the decrease in DM with increasing dose of clethodim herbicide for the resistant and susceptible ryegrass biotypes, which were more pronounced for the SUS (Figure 3).

The dose required to reduce 50% of DM (GR_{50}) was 0.2 g a.i. ha^{-1} of clethodim for the susceptible biotype and 108 and 115 g a.i. ha^{-1} of clethodim, respectively, for the resistant biotypes COQ 6 and COQ 7. Considering the values of RF 540 and 575 for biotypes COQ 6 and COQ 7, respectively, confirms that the assessed biotypes are resistant to the herbicide clethodim (Table 2). For *Lolium multiflorum*-resistant biotype to ACCase inhibitor herbicides showed a value of RF 400 for the herbicide diclofop and 165 for the herbicide clodinafop (Kuk et al., 2008).

The isolated application of clethodim herbicide at 14 DAHA provided control of approximately 52% for the SUS, but lower values for the resistant biotype COQ 8 (Table 3). However, at 28 DAHA, the SUS biotype control evolved to approximately 71%, while the resistant biotype was still lesser at 39%. The application of the cyt-P450 inhibitor PBO alone did not show any difference among the evaluated biotypes.

The association of clethodim and PBO resulted in

Table 1. Values of C_{50} with confidence intervals (CI) and resistance factor (RF) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose (0, 13,5, 27, 54, 108, 216, 432 e 864g a.i. ha^{-1}) evaluated at 20 and 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013.

Biotype	**C_{50}[1]**	**CI**	**RF²**
	g a.i. ha^{-1}	**95%**	
	20 DAHA		
COQ 6	520	208.4 – 330.6	30.5
COQ 7	410	170.9 – 387.1	24.11
SUS	17	16.3 – 17.7	-
	32 DAHA		
COQ 6	270	211.3 – 328.7	28.4
COQ 7	280	166.8 – 393.1	29.5
SUS	9.5	8.8 – 10.2	-

[1]C_{50} = dose that provide 50% of population control; [2]Resistance factor of *Lolium multiflorum* resistant biotypes, obtained by division of C_{50} of resistant biotype in relation to susceptible biotype to clethodim.

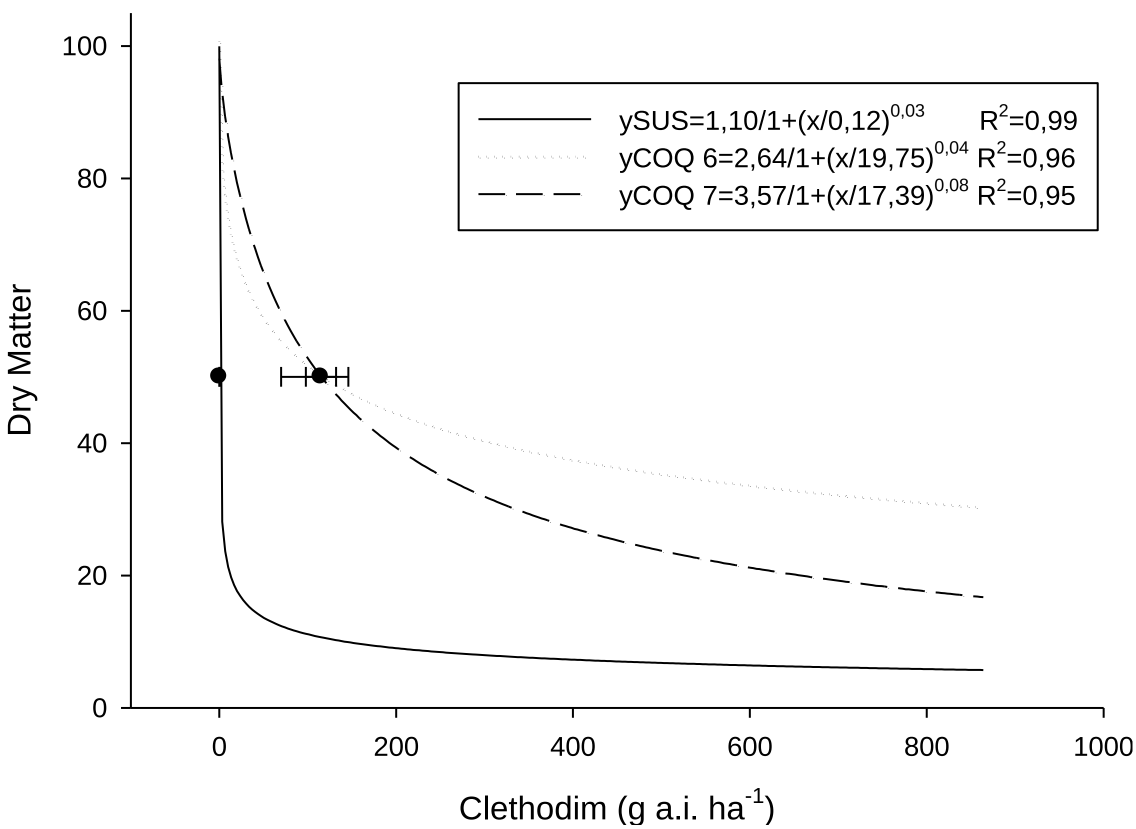

Figure 3. Dry matter production (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 32 days after herbicide applications (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of dry matter reduction.

Table 2. Values of clethodim concentration that reduced above-ground dry matter by 50% (GR_{50}) with confidence intervals (CI) and resistance factor (RF) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose (0, 13.5, 27, 54, 108, 216, 432 e 864 g a.i. ha^{-1}), evaluated at 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013.

Biotype	GR_{50}[1]	CI	RF[2]
	g i.a. ha^{-1}	95%	
COQ 6	108	70.1 – 145.9	540
COQ 7	115	97.9 – 132.1	575
SUS	0.2	0.1 – 0.3	-

[1]GR_{50} = dose that provide 50% of dry matter production; [2]Resistance factor of *Lolium multiflorum* resistant biotypes, obtained by division of C_{50} of resistant biotype in relation to susceptible biotype to clethodim.

Table 3. Control (%) at 14 and 28 days after herbicide application (DAHA) and dry matter production (g) of *Lolium multiflorum* biotypes resistant (COQ 8) and susceptible (SUS), submitted to clethodim application, alone or preceding in thirty minutes of the application of cyt-P450 monooxigenase inhibitor (PBO) and control without application. Capão do Leão/RS, 2014.

Treatments	Biotypes					
	SUS			COQ 8		
	Control (%) at 14 DAHA[1]					
Control	A[2]	0.0	b[2]	A	0.0	c
PBO	A	1.2	b	A	2.5	c
Clethodim	A	52.5	a	A	33.7	b
PBO + Clethodim	A	48.7	a	B	45.2	a
C.V (%)			18.64			
	Control (%) at 28 DAHA					
Control	A	0.0	b	A	0.0	c
PBO	A	1.0	b	A	0.0	c
Clethodim	A	71.5	a	B	39.5	b
PBO + Clethodim	A	75.0	a	A	77.0	a
C.V (%)			9.44			
	Dry matter production (g $plant^{-1}$)					
Control	A	3.705	a	A	3.785	a
PBO	A	3.332	a	A	3.215	a
Clethodim	B	1.452	b	A	2.985	a
PBO + Clethodim	A	0.925	b	A	1.307	b
C.V (%)			22.39			

[1]Days after herbicide application. [2]Means followed by the same letter in the lines (uppercase) and in the columns (lowercase), does not differ significantly by Tukey's test ($p \leq 0.05$).

increased control in biotype COQ 8 at 14 and 28 DAHA. The control values were 48 and 75% for SUS biotype and 45 and 77% for COQ 8 biotypes at 14 and 28 DAHA, respectively. Thus, the PBO increased the phytotoxicity of herbicide-resistant biotype at the same level as that for SUS, resulting in a synergistic effect. Biotypes of *Lolium* *rigidum* and *Alopecurus myosuroides* showed better control by the herbicide chlorotoluron and fenoxaprop-ethyl, respectively, when subjected to a prior application of PBO (Burnet et al., 1993; Yu et al., 2014a).

At 14 and 28 DAHA, the application of clethodim alone and clethodim + PBO affected distinctly the COQ 8 biotype, as for these treatments, the control was close to 33 and 45% at 14 DAHA and 39% and 77% at 28 DAHA, respectively, (Table 3). In biotypes of *A. myosuroides*, the addition of PBO inhibitor reduced the resistance factor for the fenoxaprop-ethyl herbicides clodinafop and haloxyfop (Letouze and Gasquez, 2003).

In the DM variable it was observed that in both biotypes the isolated application of PBO did not reduce in comparison with the control (Table 3). In the isolated application of clethodim, the SUS reduced DM on 61% reduction in comparison with the control, while, for biotype COQ 8, the reduction was not significant compared to control treatment. However, the application of PBO+clethodim resulted in 56% reduction of DM in resistant biotype as compared to isolated application of the herbicide, which resulted in a synergistic effect by the association (Table 3). These results corroborate to the control results at 14 and 28 DAHA, when the application of clethodim, preceded by the application of the cyt P450 inhibitor PBO induced biotype control increment of resistance in COQ 8, resulting in a synergistic effect.

Analyses of DM revealed that the application of clethodim preceded by PBO application resulted in lower DM as compared to other treatments for biotype COQ 8 (Table 3). *E. phyllopogon* plants previously treated with inhibitor of cyt-P450 showed an increase in control and lower fresh weight as compared to plants treated only with penoxsulam, suggesting possible involvement of metabolism as a resistance mechanism (Yasuor et al., 2009).

Among the major enzyme systems, P450 monooxygenase (cyt P450) and GST have greater importance for metabolism of herbicides as a cause of resistance. However, the expression of these enzymes which confer resistance by metabolization can be influenced by environmental factors or by epigenetic factors, which do not result in the passage of this trait to the progeny (Milder et al., 2007; Gressel, 2009).

Biotypes of *Lolium multiflorum* evaluated in this study showed changes in the metabolism pattern, which can explain the mechanism of resistance to the herbicide clethodim observed in Brazilian south fields. The increased control and reduction of DM in the resistant biotype submitted to clethodim + PBO compared with clethodim alone, indicates that the resistance of these biotypes to ACCase inhibitor herbicide (clethodim) could be due to the increased P450 enzyme activity as verified in an early work conducted by Yu et al. (2013). However, other studies about metabolism of clethodim in ryegrass need to be conducted to be performed aimed at obtaining more concrete confirmation of the results with the use of other inhibitors of cyt-P450, as different inhibitors act by inhibiting the P450 isoenzymes differently.

Conclusion

The ryegrass biotypes COQ 6 and COQ 7 are resistant to the herbicide clethodim. The Resistance Factor for COQ 6 and COQ7 biotypes were 28.4 and 29.5, with 50% reduction of DM with doses 540- and 575-times greater, respectively, than that necessary for biotype susceptible. The PBO reverses the insensitivity of ryegrass biotype COQ 8 to clethodim herbicide, indicating that metabolism is the likely cause of insensitivity of this biotype to clethodim.

Conflict of Interests

The authors have not declared any conflict of interests.

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Prevention of yield losses caused by glyphosate in soybeans with biostimulant

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Enhanced selectivity can be achieved for some herbicide treatments through the application of chemicals that reduce or protect plants from injuries. The objective of this work was to evaluate the potential to prevent herbicide yield losses in glyphosate-resistant soybean by the use of the biostimulant Fertiactyl PÓS. Based on results from two field experiments, the association of Fertiactyl PÓS with glyphosate applied post emergence of soybeans reduced foliar injuries and prevented yield losses caused by the herbicide, either when glyphosate was applied in one single application or when it was applied in two sequential applications. However, the application of the Fertiactyl PÓS was not enough to assuage the injuries caused by the mixtures of glyphosate+lactofen and glyphosate+chlorimuron-ethyl.

Key words: *Glycine max*, mitigation, selectivity.

INTRODUCTION

Weed control is intended to reduce or eliminate interference with crop of interest. The development of glyphosate-resistant (GR) genetically modified soybeans resulted in an alternative chemistry for farmers to deal with emerged weeds during crop cycle, mainly due to the wide spectrum of glyphosate, its systemic effect and economic viability (Ferreira et al., 2013). Despite the large adoption of GR crops and the substantial increase in glyphosate use, tolerant and resistant weeds still pose a challenge in weed management, requiring, many times, multiple sequential applications or the association of glyphosate with other herbicides (Ferreira et al., 2009).

Such herbicide treatments are usually more prone to cause injuries in soybeans due to the combined effect of herbicides, leading to decreased selectivity for the crop (Serra et al., 2011). Many farmers have observed that some GR soybean cultivars exhibit visually perceived injuries shortly after post emergence applications of glyphosate (Santos et al., 2007; Zablotowicz and Reddy, 2007). A typical symptom observed in the field after glyphosate application on GR soybeans is the yellowing of the upper leaves known as “yellow flashing”.

The chlorotic symptoms in soybeans probably reflects lower chlorophyll content in glyphosate-treated plants as

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a result of direct damage to chlorophyll by glyphosate (Reddy et al., 2004; Zobiole et al., 2010a, b, 2012) or immobilization of Mg and Mn (Zobiole et al., 2010a, b), nutrients required for chlorophyll production and function. The main metabolite of glyphosate, aminomethyl-phosphonic acid (AMPA), may also cause injury to GR-soybeans treated with glyphosate and contribute to chlorosis (Duke et al., 2003; Reddy et al., 2004). Previous studies have also demonstrated that glyphosate or one of its metabolites can also directly affect nodulation (De Maria et al., 2006; Zobiole et al., 2010c). Reduced nodulation is associated to the direct effect on symbiotic species and/or to Ni depletion (Zobiole et al., 2010c), since Ni is both an essential element for microbial nitrogen fixation (Ureta et al., 2005). Ni deficiency symptoms in soybeans may also include chlorosis, due to lower absorption of iron.

Biostimulants are substances and/or microorganisms used to potencialize plant growth, usually enhancing the plant's ability to assimilate applied nutrients or providing benefits to plant development. The definition of plant biostimulants is still evolving, which is partly a reflection of the diversity of inputs that can be considered stimulants (Calvo et al., 2014).

Recent reviews support the potential of different types of biostimulants to improve plant biomass, crop yield and resistance to multiple types of stress (Calvo et al., 2014; Nardi et al., 2016). So far, no focus has been given to the hypothesis that biostimulants may also have an impact on preventing herbicide toxicity on cultivated species.

Herbicide application represents an abiotic stress, due to secondary effects related to metabolization in plan tissues and to the recovery from physiological injuries. The selectivity of some herbicides may, therefore, be enhanced with biostimulants. Fertiactyl Pós™ is a foliar-applied product containing a patented biostimulant complex, and its technology enables rapid and efficient nutrient absorption by the plant, ensures an active transfer of nutrients through the plant and stimulates physiological activity in the areas of stress resistance, root development and increased photosynthesis (Santos et al., 2015).

As mentioned earlier, this study aimed to evaluate the potential of a biostimulant to prevent damages in soybean plants caused by post emergence applications of herbicides.

MATERIALS AND METHODS

Two field experiments were carried out at Mandaguaçu, PR (Brazil) (23°14'28"S; 52°00'23"W), from November 2014 to March 2015.

Main soil properties in the experimental area were pH (H_2O) = 6.0; CEC = 8.41 $cmol_c\ dm^{-3}$; $H^+ + Al^{3+}$ = 2.5 $cmol_c\ dm^{-3}$; Ca^{2+} = 4.3 $cmol_c\ dm^{-3}$; Mg^{2+} = 1.3 $cmol_c\ dm^{-3}$; K^+ = 0.31 $cmol_c\ dm^{-3}$; P = 13 mg dm^{-3}; Organic matter = 3.1%; sand = 26.3%; silt = 14.5% and clay = 59.2%.

Monsoy 6210 IPRO soybean variety was sown on 11/07/2014, with the application of 200 kg ha^{-1} of NPK 00-20-18 fertilizer. Row spacing was 0.45 cm and sowing density was 12 seeds m^{-1}, aiming a final population of 250.000 plants ha^{-1}. Soybean emergence occurred five days after sowing.

Two simultaneous experiments were carried out, the first with a single application of the herbicide treatments (Experiment 1) and the second one with two sequential applications (Experiment 2). Treatments for both experiments are described in Table 1.

Due to the proprietary nature of the product (Fertiactyl Pós™) investigated, detailed information cannot be present on its overall composition. Its composition includes 1% of manganese complexed by heteroatom-stabilized complex (HSC) and humic and fulvic acids from seaweed extracts, but its mode of action is currently unknown.

The post emergence application of treatments in experiment 1 was performed when soybean was at V4/V5 stage (4 to 5 trifoliates leaves), and environmental conditions for the application were average (avg.) temperature of 23°C, average air moisture of 70%, wind speed of 1.0 km h^{-1}, moist soil and clear sky.

The first post emergence application of treatments for experiment 2 was performed when soybean was at V2/V3 stage (avg. temp. = 24°C, avg. air moisture = 69%, wind speed = 1.1 km h^{-1}, moist soil and few clouds) and the second sequential application was performed at V5 (avg. temp. = 24°C, avg. air moisture = 75%, wind speed =1.2 km h^{-1}, moist soil and cloudy sky).

For all applications, a CO_2-pressurized backpack sprayer under constant pressure (2.46 kgf cm^{-2} – 35 PSI) equipped with three XR 11002 flat-fan tips was utilized, providing an application volume of 200 L ha^{-1}. Both experiments were kept free of weeds by periodical hoeing.

Each experimental unit consisted of eleven 4-m long soybean rows. In the evaluations, 0.5 m of each end of the plots and the border rows were not considered, totaling an effective area of 12.1 m^2. Climatic data (temperature and precipitation) during the field conduction of this work are as in Figure 1.

In experiment 1, soybean injuries were evaluated at 7 and 14 days after application (DAA) through the visual scale proposed by the European Weed Research Council (EWRC, 1964), where 1 means no damage and 9 means death of all plants. Plant height evaluations were also performed at 7, 14 and 42 DAA, by measuring 10 plants per plot from soil to the insertion of the last fully expanded trifoliate. In experiment 2, soybean injury evaluations were performed at 7 and 14 days after the first application (DAA-A) and at 7 and 14 days after the second sequential application (DAA-B). Plant height evaluations at 14 DAA-A and at 7, 14 and 42 DAA-B were also conducted.

To determine soybean grain yield, all plants in the effective area were manually harvested (03/04/14), threshed, and the grains were later separated from impurities and weighed. Samples for moisture determination (portable moisture analyzer model Mini GAC) were taken from each plot and grain moisture was corrected to 13%. Harvest from each plot was sampled in triplicates to perform the hundred-grains weight (HGW).

For both experiments, the experimental design was randomized blocks with seven treatments and four replications. The plots were arranged with "twofold" checks, that is, with two adjacent, non-treated checks for each treated plot. Such field arrangement has been previously used and detailed in other previous selectivity screenings (Fagliari et al., 2001; Constantin et al., 2007). The focus of all comparisons in this work is between each treatment *versus* non treated "twofold" checks (control) rather than comparison among herbicide treatments. All data were subjected to analysis of variance and the averages of the significant variables were compared by the F-test ($p \leq 0.10$).

RESULTS AND DISCUSSION

Glyphosate application resulted in soybean injuries

Table 1. Description of herbicide treatments with products and doses evaluated in experiments 1 and 2. Mandaguaçu (PR), 2014/2015.

Treatment[1/]	Dose of the herbicides (g ha^{-1} of active ingredient)	Abbreviations
Experiment 1 (Single post emergence application at V4/V5)		
Glyphosate	1944	GLY
FP	400[2]	FP
Glyphosate+lactofen	1944+72	GLY+LAC
Glyphosate+chlorimuron-ethyl	1944+15	GLY CHL
Glyphosate+FP	1944+400	GLY+FP
Glyphosate+lactofen+FP	1944+72+400[2/]	GLY+LAC+FP
Glyphosate+chlorimuron-ethyl+FP	1944+15+400[2/]	GLY+CHL+FP
Experiment 2 (Two sequential post emergence applications at V2/V3 and at V5)		
Glyphosate/Glyphosate	960 / 960	GLY / GLY
FP/FP	400[2/] / 400[2/]	FP / FP
Glyphosate/Glyphosate+lactofen	960 / 960+72	GLY / GLY+LAC
Glyphosate/Glyphosate+chlorimuron-ethyl	960 / 960+15	GLY / GLY+CHL
Glyphosate+FP/Glyphosate+FP	960+400[2/] / 960+400[2/]	GLY+FP / GLY+FP
Glyphosate+FP/Glyphosate+lactofen+FP	960+400[2/] / 960+72+400[2/]	GLY+FP / GLY+LAC+FP
Glyphosate+FP /Glyphosate+chlorimuron-ethyl+FP	960+400[2/] / 960+15+400[2/]	GLY+FP / GLY+CHL+FP

The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Glyphosate = Roundup Ready™; Lactofen = Cobra™; Chlorimuron-ethyl = Classic™; FP = Fertiactyl PÓS™. [2/] Dose of the biostimulant Fertiactyl PÓS in mL ha^{-1} of commercial product.

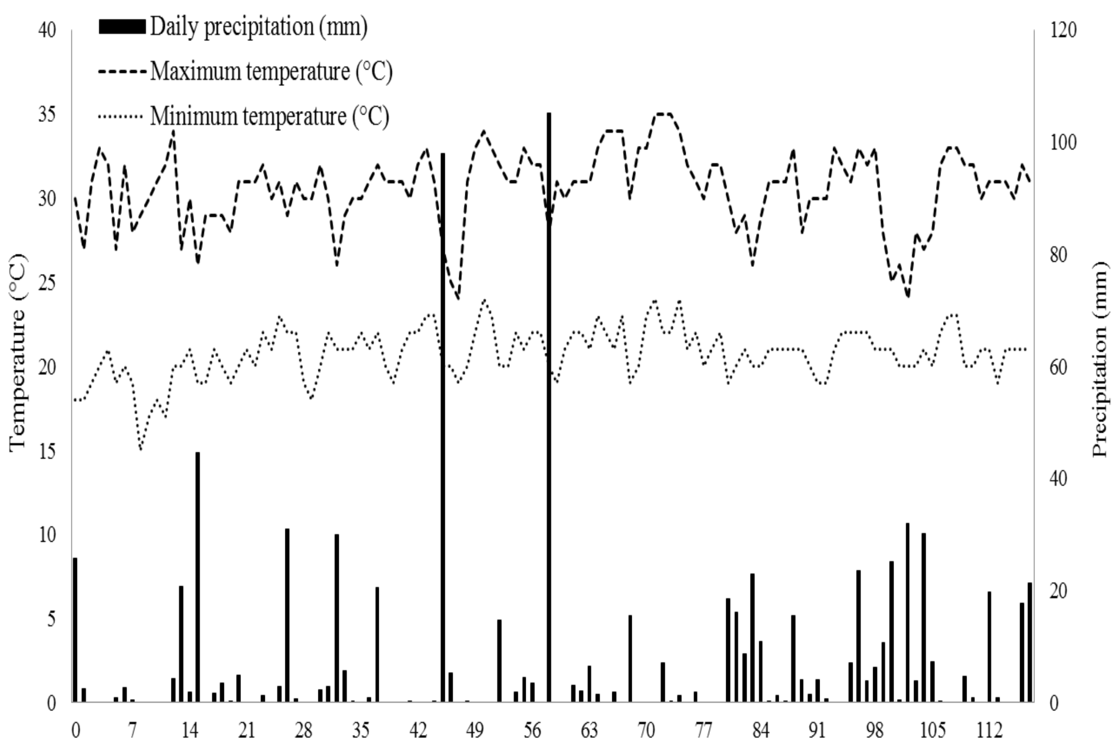

Figure 1. Daily precipitation, maximum temperature and minimum temperature during the period when the experiments were carried out. Mandaguaçu (PR), 2014/2015.

Table 2. Soybean injury based on EWRC scale (EWRC, 1964) and plant height after application of different combinations of herbicides and biostimulant (experiment 1). Mandaguaçu (PR), 2014/2015.

Treatment	Soybean injury (EWRC Scale, 1-9)				Plant Height (cm)								
	7 DAA		14 DAA		7 DAA			14 DAA			42 DAA		
	Treat.[1/]	Contr.[2/]	Treat.[1/]	Contr.[2/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]
GLY	2	1	1	1	16.46	15.78	-0.68	31.17	30.83	-0.34	86.15	88.32	2.17
FP	1	1	1	1	15.50	16.00	0.50	32.14	30.01	-2.13*	87.35	87.42	0.07
GLY+ LAC	5	1	1	1	15.82	16.37	0.55	28.92	31.57	2.65*	83.45	88.72	5.27*
GLY+CHL	5	1	1	1	15.75	16.14	0.39	30.78	32.37	1.59	86.55	90.42	3.87
GLY+FP	2	1	1	1	14.67	15.92	1.25	32.17	34.39	2.22*	88.65	91.50	2.85
GLY+LAC+FP	5	1	1	1	15.00	15.28	0.28	29.64	30.33	0.69	84.45	88.12	3.67
GLY+CHL+FP	5	1	1	1	14.46	15.17	0.71	29.85	29.89	0.04	84.90	83.47	-1.43
CV (%)	–		–			7.68			7.56			6.19	

GLY: Glyphosate; FP: Fertiactyl PÓS™; LAC: lactofen; CHL: chlorimuron-ethyl. The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Treat. = treatments. [2/]Contr. = Control (average of two fold checks). [3/]Difference between control and treated plants. *Significant differences between treatment and control by the F-test ($p \leq 0.10$).

(Tables 2 and 3), featured by the typical yellowing of the upper leaves. Symptoms were light with GLY or GLY+FP but more evident with GLY+LAC, GLY+CH, either with or without FP (Table 2). Some GR soybean cultivars are unequivocally injured by glyphosate (Zablotowicz and Reddy, 2007). However, injuries vary depending on the soybean cultivar, on the dose, on glyphosate formulation, and on environmental factors (Zobiole et al., 2011). The duration of the yellowing depends on the plants ability to restore adequate levels of these elements by root or foliar uptake (Jolley, 2004; Eker et al., 2006).

After one single application of herbicides, visual injuries were quickly overcome and no more symptoms were found at 14 DAA (Table 2). In experiment 2, herbicide treatments produced a similar pattern of soybean injury after first (7 DAA-A) and second (7 DAA-B and 14 DAA-B) applications (Table 3).

Application of lactofen and chlorimuron-ethyl associated with glyphosate caused more severe injuries in soybeans, with development of necrosis beyond chlorotic spots on leaves. The addition of FP to these herbicide mixtures provided no attenuation of such visual injury symptoms, both by one (Table 2) and by two sequential applications of herbicides (Table 3).

For single applications (Exp. 1), the only treatment that affected soybean growth consistently in more than on evaluation was GLY+LAC (Table 2). For sequential applications (Exp. 2), all treatments containing either lactofen or chlorimuron consistently affected plant height after second application (Table 4). Previous selectivity works with herbicide mixtures containing glyphosate have also reported that lactofen may cause more severe soybean injuries as compared to other herbicides (Almeida Jr. et al., 2010; Alonso et al., 2013). Addition of FP to GLY+LAC prevented the slower growth observed after one single application of herbicides (Table 2), but it was not effective for sequential applications of GLY/GLY+LAC and GLY/GLY+CHL (Table 4). Since the second application in experiment 2 was performed in a more advanced stage of the crop development, there could have been an insufficient period of time for the crop to recover its vegetative growth before the change of sink-source balance due to the pods and grains formation, even with the use of FP.

Soybean grain yield decreased by 6 to 9% with a single application of GLY and with all mixtures containing glyphosate except GLY+FP. Use of FP alone did not provide any increase in soybean yield, but its use in tank mixture with glyphosate prevented reduction of crop yield observed in treatment GLY. No effect of treatments was found for HGW (Table 5).

A similar effect of glyphosate in soybean yield was found for sequential applications of herbicides in experiment 2. Two sequential applications of glyphosate (GLY / GLY) reduced crop yield in ≈10%, but the inclusion of FP in both applications (GLY+FP / GLY+FP) prevented yield losses.

The effect of FP is probably not directly related

Table 3. Soybean injury based on EWRC scale (EWRC, 1964) after application of different combinations of herbicides and biostimulant (experiment 2). Mandaguaçu (PR), 2014/2015.

Treatments	Soybean injury (EWRC Scale, 1-9)							
	7 DAA-A		14 DAA-A		7 DAA-B		14 DAA-B	
	Treat.[1/]	Contr.[2/]	Treat.[1/]	Contr.[2/]	Treat.[1/]	Contr.[2/]	Treat.[1/]	Contr.[2/]
GLY / GLY	2	1	1	1	1.75	1	2	1
FP / FP	1	1	1	1	1	1	1	1
GLY / GLY+LAC	5	1	1	1	5	1	5	1
GLY / GLY+CHL	5	1	1	1	5	1	5	1
GLY+FP / GLY+FP	2	1	1	1	1.5	1	2	1
GLY+FP / GLY+LAC+FP	5	1	1	1	4	1	5	1
GLY+FP / GLY+CHL+FP	5	1	1	1	5	1	5	1

GLY: Glyphosate; FP: Fertiactyl PÓS™; LAC: lactofen; CHL: chlorimuron-ethyl. The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Treat. = treatments. [2/]Contr. = Control (average of two fold checks).

Table 4. Effect of two sequential applications on soybean height with different combinations of herbicides and biostimulant (experiment 2). Mandaguaçu (PR), 2014/2015.

Treatments	Plant Height (cm)											
	14 DAA-A			7 DAA-B			14 DAA-B			42 DAA-B		
	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]
GLY / GLY	18.42	18.10	-0.32	32.46	32.33	-0.13	42.83	44.42	1.59	99.90	101.32	1.42
FP / FP	17.57	18.07	0.50	32.21	33.03	0.82	49.23	45.75	-3.48*	104.10	104.37	0.27
GLY / GLY+LAC	16.89	16.71	-0.18	27.42	32.91	5.49*	37.96	45.10	7.14*	92.30	102.15	9.85*
GLY / GLY+CHL	17.46	17.83	0.37	26.78	31.71	4.93*	37.89	45.20	7.31*	90.35	101.37	11.02*
GLY+FP / GLY+FP	18.35	17.80	-0.55	31.03	32.39	1.36	43.53	44.55	1.02	101.10	103.57	2.47
GLY+FP / GLY+LAC+FP	17.92	17.71	-0.21	28.39	33.33	4.94*	38.92	44.06	5.14*	91.40	101.62	10.22*
GLY+FP / GLY+CHL+FP	17.42	17.85	0.43	26.25	32.55	6.30*	35.64	43.12	7.48*	92.00	103.85	11.85*
CV (%)		8.36			8.41			5.43			4.54	

GLY: Glyphosate; FP: Fertiactyl PÓS™; LAC: lactofen; CHL: chlorimuron-ethyl. The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Treat.: Treatments. [2/]Contr.: control (average of two fold checks). [3/]Difference between control and treated plants. *Significant differences between treatment and control by the F-test ($p \leq 0.10$).

to plant nutrition, since two sequential applications (FP / FP) did not provide any yield gain (Table 6). Despite the positive preventive effect of FP, no effect was found in relation to yield losses caused by sequential applications of herbicide mixtures in experiment 2. In general, grain losses for sequential applications ranged from 11 to 15% and were more relevant than those found for single applications in Exp. 1. Similarly to Exp. 1, no effects were found on HGW (Table 6), indicating that other yield components such as number of pods per plant or number of seeds per pod may have been affected.

The reduction of glyphosate selectivity in GR soybeans when it is applied in tank mixtures with

Table 5. Hundred grains weight (HGW) and soybean grain yield after application of different combinations of herbicides and biostimulant (experiment 1). Mandaguaçu (PR), 2014/2015.

Treatments	HGW (g)			Yield (kg ha^{-1})			
	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Pr > F[4/]
GLY	13.77	13.70	-0.07	4133.31	4419.37	286.05*	0.064
FP	13.41	13.70	0.29	4439.71	4298.40	-141.30	0.345
GLY+LAC	13.83	14.02	0.19	3952.62	4251.40	298.78*	0.054
GLY+CHL	14.03	14.02	-0.01	3971.05	4383.95	412.90*	0.010
GLY+FP	14.59	13.96	-0.63	4528.91	4601.31	72.40	0.625
GLY+LAC+FP	14.02	14.12	0.10	4307.22	4635.84	328.61*	0.035
GLY+CHL+FP	14.01	13.34	-0.67	4130.20	4401.76	271.56*	0.077
CV (%)		4.82			6.66		

GLY: Glyphosate; FP: Fertiactyl PÓS™; LAC: lactofen; CHL: chlorimuron-ethyl. The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Treat.: Treatments. [2/]Contr.: control (average of two fold checks). [3/]Difference between control and treated plants. [4/]Probability of F being significant. *Significant differences between treatment and control by the F-test ($p \leq 0.10$).

Table 6. Hundred grains weight (HGW) and soybean grain yield after application of different combinations of herbicides and biostimulant (experiment 2). Mandaguaçu (PR), 2014/2015.

Treatments	HGW (g)			Yield (kg ha^{-1})			
	Treat.[1/]	Contr.[2/]	Diff.[3/]	Treat.[1/]	Contr.[2/]	Diff.[3/]	Pr > F[4/]
GLY / GLY	14.69	14.40	-0.29	4146.46	4584.78	-438.32*	0.041
FP / FP	13.58	13.95	0.37	4612.48	4622.85	-10.37	0.960
GLY / GLY+LAC	14.17	14.07	-0.10	4056.87	4751.04	-694.17*	0.002
GLY / GLY+CHL	14.16	14.16	0.0	4123.16	4625.12	-501.96*	0.021
GLY+FP / GLY+FP	14.42	14.35	-0.07	4544.27	4678.38	-134.11	0.513
GLY+FP / GLY+LAC+FP	13.80	14.27	0.47	3968.58	4659.51	-690.93*	0.002
GLY+FP / GLY+CHL+FP	14.46	14.12	-0.34	3983.63	4545.14	-561.51*	0.011
CV (%)		5.71			5.67		

GLY: Glyphosate; FP: Fertiactyl PÓS™; LAC = lactofen; CHL: chlorimuron-ethyl. The sign + indicates tank mix; the sign / indicates a sequential application. [1/]Treat.: treatments. [2/]Contr.: control (average of two fold checks). [3/]Difference between control and treated plants. [4/]Probability of F being significant. *Significant differences between treatment and control by the F-test ($p \leq 0.10$).

lactofen and chlorimuron-ethyl found in both experiments in this report has also been described by several authors (Santos et al., 2007; Alonso et al., 2010; Zobiole et al., 2010c, d, 2011). Lower selectivity in these cases was attributed to combined negative effects on nodulation, soil microbiota, photosynthesis and efficient use of water in the soil, as well as in changes in levels of some nutrients present in the leaves and less vegetative growth.

The application of FP in combination with glyphosate and the sequential applications of GLY+FP / GLY+FP were treatments that prevented yield losses caused by glyphosate. However, there was no prevention of yield loss due to the application of GLY+LAC and GLY+CHL in combination with FP in both experiments. The difference between both situations could be the type of injuries caused by the herbicide treatments, more transitory for GLY alone but more long-lasting for the necrotic lesions on leaves caused by GLY+LAC and GLY+CHL (Alonso et al., 2013).

Since the mode of action of FP is unknown, all theories to support the preventive damage effect are speculative. However, one possible explanation could be the fact that some biostimulants can enhance photosynthesis (Giannattasio et al., 2013) what could serve as a reparative effect of the already reported reduction of GR soybean photosynthesis following application of glyphosate (Zablotowicz and Reddy, 2007; Zobiole et al., 2011; 2012).

Another possibility is that FP components such as humic and fulvic acids may have an effect on amelioration of the abiotic stress caused by glyphosate application. The stimulatory effects of humic and fulvic acids in some studies result in enhanced tolerance to salinity stress (Ertani et al., 2013) and drought tolerance (García et al., 2012; Anjum et al., 2011). Furthermore, humic substances have been reported to enhance some aspect of growth in several species of plants, including soybeans (Calvo et al., 2014).

Conclusions

The association of FP with glyphosate in post emergence of GR soybeans prevented yield losses caused by this herbicide, either when glyphosate was applied in one single application or when it was applied in two sequential applications. However, the application of FP was not enough to assuage the injuries caused by the mixtures of glyphosate+lactofen and glyphosate+ chlorimuron-ethyl.

Conflict of Interests

The authors have not declared any conflict of interests.

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Assessment of the levels of cadmium and lead in soil and vegetable samples from selected dumpsites in the Kumasi Metropolis of Ghana

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Many dumpsites in the urban communities in Ghana are used for cultivation of crops, especially vegetables. However, these dumpsites may serve as potential sources of soil heavy metals that could enter the food chain mainly through cultivated food crops with serious consequences on human health. This study investigated the levels of two heavy metals, lead (Pb) and cadmium (Cd), in the soil and tissues of vegetables grown on such dumpsites. Soil and tissue (lettuce, cabbage, spring onion, tomato and the leaves of *Xanthosoma sagitifolium*) samples were collected from ten locations with two of these locations used as control. The samples were acid-digested and the metal concentrations determined using atomic absorption spectrometry. Pb and Cd contents of soils from all the eight dumpsites and one of the control locations were above the guidelines recommended by FAO and WHO. The highest Cd level in the soil (13.6 $mgkg^{-1}$ of Cd) was found at Aketego dumpsite and the highest soil Pb (36.1 $mgkg^{-1}$ Pb) was recorded at Meduma dumpsite. The leafy vegetables, cabbage, lettuce and *X. sagitifolium* (locally called *'kontomire'*) recorded relatively higher amounts of Pb and Cd in the edible parts. Further studies are required to determine how much of the daily diet these vegetables contribute to the total diets of the population and special attention to calculating the overall daily doses of Cd and Pb to pregnant mothers and children <5 years of age is thus warranted.

Key words: Cadmium (Cd), dumpsites, heavy metals, lead (Pb), health risk, vegetables.

INTRODUCTION

The swelling of urban populations in Ghana reflects rural-urban migration (Black et al., 2011) and high population growth rate in the cities (Grimm et al., 2008), impacting severely on the availability of arable lands in the urban centres (Akudugu et al., 2012). In Ghana, construction of roads and buildings has been blamed for losses of otherwise agricultural lands (Kugelman, 2012).

The agricultural prowess of some of the urban residents, the high cost of living (Aguda, 2009) and the very high unemployment (Barrios et al., 2006) in most Ghanaian

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cities have over the years promoted backyard farming (Appeaning, 2010). Many families living in these urban communities now depend upon backyard farming (Zezza and Tasciotti, 2010), which includes both livestock and crops (Cofie et al., 2005). Urban agriculture contributes about 20% of Ghana's national agricultural production (Appeaning, 2010), although about 45% of the backyard farm produce is for subsistence. Unfortunately, due to the pressure on land use in the urban settings, farmers tend to use unapproved land sites for agricultural purposes (Egyir and Beinpuo, 2009), including abandoned refuse dump sites (Agyarko et al., 2010). Dumpsites are commonly used for direct cultivation of vegetables and also as good source of compost to support mainland agricultural activities (Owusu-Sekyere et al., 2013; Mwingyine, 2008).

Despite the value of plant-derived compost and soils in refuse dumps (Stefanowicz et al., 2012), many hazardous chemicals are found in these dump sites, and consequently, they pose threat to human health when they become introduced to the food chain (Bagumire et al., 2009). Because metals and other inorganic chemicals are not degraded, they can occur in elevated concentrations with time due to accumulation. Some plastics, papers, batteries, electric bulbs and bottle caps, are known to contain heavy metals (Amusan et al., 2005; Akpoveta et al., 2011; Ideriah et al., 2010; Adelekan and Alawode, 2011; Kolo et al., 2014).

There is an increasing trend of industrial waste dumping in most African countries, an activity mainly motivated by importation of cheap used manufacturing products from industrial countries by these poor African nations. This menace has been identified in Ghana with serious consequences on human health and the environment (Agyarko et al., 2010). Arsenic, cadmium, lead, chromium, nickel, cobalt and mercury are of great concern primarily because of their potential to harm soil organisms, plants, animals and ultimately humans (Singh et al., 2011a). Heavy metals are taken up alongside essential elements such as zinc, magnesium and calcium by plants (Volpe et al., 2009). Research has shown that vegetables grown on refuse dump sites have tendency to absorb these heavy metals, hyper-accumulate them in their tissues with detrimental effects on plant growth and yield (Amusan et al., 2005; Nabulo et al., 2008) as well as the health of consumers (Reilly, 2008; FAO/WHO, 2011; Järup, 2003).

This study investigated the levels of two very important heavy metals, that is, Cd and Pb, in refuse dumpsites in the Kumasi Metropolis of Ghana and their accumulations in edible tissues of vegetables commonly cultivated on these farms.

MATERIALS AND METHODS

Study site

The study sampled eight refuse dump sites (Emina, Oduom, Tafo, Aketego, Ayigya Zongo, Meduma, South Suntreso and Boadi) and two control farms (KNUST Horticulture and Ayeduase Agric Farm). The refuse dumps sites at Aketego, Ayigya Zongo and Boadi were relatively young as they were established about 5 years prior to the study according to the Assemblyman of the area - vegetable cultivation began in the last three years (Personal Interview). The study sites were grouped into two clusters (that is, Clusters 1 and 2) based on the types of vegetables cultivated. Cluster 1 consisted of six refuse dump sites and a control farm that were used for cultivation of lettuce, cabbage and spring onion. Cluster 2 was for cultivation of tomatoes and leaves of cocoyam or *Xanthosoma sagitifolium* (locally called '*kontomire*'). All the vegetables from these farms are regularly sold on the local markets in the Kwame Nkrumah University of Science and Technology (KNUST), surrounding communities and across Kumasi Metropolis.

Sample collection

Soil (1.0-5.0 inches deep) and vegetables (that is, lettuce, cabbage, spring onion stems, tomato fruits and cocoyam leaves) were each collected into separate transparent, air-tight polyethylene bags and kept on ice before transporting to the laboratory for the metal analysis. Five samples each of soil and the vegetables were collected for the heavy metals determination. The period of the research spanned over three years from 2012 to 2014.

Pre-digestion of samples for heavy metal analysis

In the laboratory, samples were washed using metal-free distilled water to remove soil particles on the surfaces. The washed vegetable samples were air-dried for 2 h at room temperature and immediately followed by oven-drying at 105°C for 5 h. The dried samples were ground into fine powder using mortar and pestle and stored in clean containers. Soil samples, on the other hand, were air/sun-dried for 2 h, homogenized and sieved using 2 mm nylon mesh. The powdered soil and vegetable samples were stored in clean containers.

Acid digestion of vegetable and soil samples

Triplicates of 2 g dry weight (dw) of finely ground soil and vegetable samples were each placed in 300 ml volumetric flask, 20 ml of HNO_3-$HClO_4$ di-acid mixture (9:4) added, and the contents well mixed by swirling thoroughly. The flasks with contents were slowly heated on hot plate in a fume chamber to 85°C and then rapidly to 150°C. Heating continued until the production of red NO_2 fumes ceased and the contents' volume reduced to 3 to 4 ml of colorless or yellowish slurry. The flasks and their contents were cooled and the volumes adjusted with deionized water before instrumental analysis. The resulting solutions were kept in vials and stored at 4°C pending Atomic Absorption Spectrometry (AAS) determination. The machine was calibrated using standard samples and also blanks to ensure reliability of the test results.

Statistical analysis

The experiment was a complete randomized block design with triplications of soil and vegetable samples. Each replicate consisted of three different samples of each vegetable and soil collected randomly from the farm sites. Analysis of variance (ANOVA) for the effects of refuse dump sites and vegetable type on the mean values of Pb and Cd was tested at 5% probability level using the statistical package Graphpad Prism 4 (Graphpad Software, Inc.).

Table 1. Mean concentration ($mgkg^{-1}$) of Cd in soil and vegetable samples from refuse dump sites in the Kumasi Metropolis of Ghana.

Soil/Vegetable	Refuse dump sites							
	Emina	Oduom	Tafo	Aketego*	Ayigya Zongo*	Boadi*	Control Farm (C1)	FAO/WHO[b]
Soil	0.453(±0.04)	0.243(±0.07)	0.543(±0.04)	13.623(±0.31)	0.363(±0.03)	0.280(±0.02)	0.253(±0.02)	0.27
Lettuce	0.017(±0.01)	0.067(±0.01)	0.017(±0.01)	0.037(±0.00)	0.003(±0.00)	0.053(±0.00)	0.010(±0.01)	0.2
Cabbage	0.200(±0.02)	0.117(±0.02)	0.107(±0.02)	0.073(±0.00)	0.143(±0.03)	0.013(±0.00)	0.023(±0.00)	0.05 (brassica)
Spring onion	0.060(±0.02)	0.033(±0.02)	0.033(±0.01)	0.033(±0.00)	0.060(±0.01)	0.030(±0.01)	0.020(±0.01)	0.2

[a]C1 is the control farm±SEM. [b]Maximum levels (MLs) of Cd in leafy and fruiting vegetables according FAO/WHO (CODEXAlimentarius Committee, 2011) are 0.2 $mgkg^{-1}$ and 0.05 $mgkg^{-1}$, respectively. *Relatively younger refuse dump sites. Averaged Cd concentration in soil is 0.270$mgkg^{-1}$ (WHO/FAO: from ftp://ftp.fao.org/codex/meetings/CCCF/cccf5/cf05_INF.pdf).

RESULTS

Vegetable cultivation

Farmers cultivated specific types of vegetables per refuse dump site. Some of these vegetables thrived better on these refuse dump sites. Seven of the ten farms were involved in cultivation of lettuce, cabbage and spring onions. Tomato and cocoyam leaves or *kontomire* were produced on the remaining three farms.

Cadmium levels in agricultural soil and vegetable samples

Only three of the ten farms recorded soil Cd level below the FAO/EPA's recommended maximum limit of 0.270 $mgkg^{-1}$ (270 ppb) for food crop production (Tables 1 and 2) (Odai et al., 2008). The Cd content of soil from the Aketego dumpsite (13.623 $mgkg^{-1}$), one of the newly-established dumps, was exceptionally high (P<0.0001), nearly 13.5 times the maximum allowable limit for Cd in agricultural soil.

Lettuce, cabbage and spring onion from all the seven farms including the control contained <0.2 $mgkg^{-1}$, the FAO/WHO guideline (Table 1 and Figure 1). Although Cd in cabbage from four dump sites (Emina, Oduom, Tafo and Ayigya Zongo) was within the safe levels, the concentrations were either double than the amounts found in same vegetables from the other sites (that is, Aketego and Boadi dumpsites, and Agric Farm C1) (Table 1). On the other hand, Cd levels in *kontomire* and tomato leaves from Meduma and South Suntreso dumpsites, and KNUST Horticulture C2 (control) were above the maximum allowable levels (p<0.0001) (Table 2 and Figure 2).

The concentration ranged from 0.340 $mgkg^{-1}$ (340 ppb) to 0.790 $mgkg^{-1}$ (790 ppb), amounts in excesses of 300 to 830% the maximum level allowed for consumption (Table 2). The Cd content of the tomato fruit from the dump sites was 7 to 11 times higher than the maximum allowable level in fruiting vegetable. These measurements were comparable (P>0.05) to the control farm (KNUST Horticulture C1) which was found to be 10 times higher than the maximum level of Cd in fruiting vegetables. Clearly the concentration of Cd in the tomato leaves and fruits were different. The leaves contained about twice the amount of Cd in the fruits (Table 2).

Pb levels in agricultural soil and vegetable samples

In all the farms including the controls, significantly high amounts of Pb was recorded in the soil above the maximum limit of 0.420 $mgkg^{-1}$ (420 ppb) allowed for agriculture (Tables 3 and 4). The highest concentration of Pb (33.870 $mgkg^{-1}$) was detected in the soil from South Suntreso dump site and the lowest from the control farm (1.13 $mgkg^{-1}$). Lettuce, cabbage and spring onion grown on old dump sites (that is, Emina, Oduom and Tafo) had Pb levels exceeding the maximum level of Pb in leafy vegetables allowed for consumption (0.3 $mgkg^{-1}$). These were about 1.5 to 17 times greater than the maximum level of Pb in leafy vegetables allowed for human consumption. However, Pb levels of lettuce, cabbage and spring onion samples from newly established dump sites (Aketego, Ayigya Zongo and Boadi) and the control (Agric Farm C1) all fell below the maximum level allowed for consumption (Table 3 and Figure 3). *Kontomire* and tomato leaf samples from Meduma, South Suntreso and KNUST Horticulture C2 (control) all recorded Pb levels 8 to 13.5 times higher than the maximum level of Pb in leafy vegetables allowed for

Table 2. Mean concentrations (mgkg^{-1}) of Cd in soil and vegetable samples from refuse dump sites in the Kumasi Metropolis of Ghana.

Soil/Vegetable samples	Refuse dump sites			FAO/WHO
	Meduma	South Suntreso	KNUST Horticulture (C2)	
Soil	0.317(±0.02)	0.617(±0.05)	0.140(±0.02)	0.27
Tomato leaf	0.873(±0.02)	0.753(±0.04)	0.790(±0.03)	0.2
Kontomire leaf	0.507(±0.03)	0.563(±0.02)	0.537(±0.05)	0.2
Tomato fruit	0.527(±0.01)	0.340(±0.01)	0.493(±0.04)	0.05

C2 is the control farm. Maximum levels (MLs) of Cd in leafy and fruiting vegetables according FAO/WHO (CODEX Alimentarius Committee, 2011) are 0.2 and 0.05 mgkg^{-1}, respectively. Average Cd concentration in soil is 0.270 mgkg^{-1} ±SEM.

Table 3. Mean concentration (mgkg^{-1}) of Pb in soil and vegetable samples from refuse dump sites in the Kumasi Metropolis of Ghana.

Soil/Vegetable samples	Refuse dump sites						
	Emina	Oduom	Tafo	Aketego*	Ayigya Zongo*	Boadi*	Agric Farm (C1)
Soil	6.040(±0.04)	10.650(±0.27)	14.617(±0.272)	23.477(±0.32)	3.843(±0.12)	1.953(±0.06)	1.133(±0.11)
Lettuce	3.533(±0.24)	7.197(±0.38)	0.933(±0.038)	0.130(±0.02)	0.063(±0.03)	0.013(±0.00)	0.030(±0.01)
Cabbage	2.433(±0.22)	0.700(±0.05)	0.577(±0.041)	0.060(±0.02)	0.127(±0.02)	0.050(±0.01)	0.040(±0.01)
Spring onion	0.780(±0.07)	0.590(±0.04)	5.367(±0.12)	0.117(±0.01)	0.037(±0.02)	0.080(±0.01)	0.070(±0.02)

C1 is the control farm. Maximum levels (MLs) of Pb in leafy and fruiting vegetables according FAO/WHO (CODEX Alimentarius Committee, 2011) are 0.3 and 0.1 mgkg^{-1} respectively. FAO Averaged Cd concentration in soil is 0.420mgkg^{-1} ±SEM

Table 4. Mean concentrations (mgkg^{-1}) of Pb in soil and vegetable samples from refuse dump sites in the Kumasi Metropolis of Ghana.

Soil/Vegetable samples	Refuse dump sites		
	Meduma	South Suntreso	KNUST Horticulture (C2)
Soil	36.120(±1.163)	33.870(±1.452)	2.540(±0.078)
Tomato leaf	4.503(±0.332)	4.517(±0.283)	5.700(±0.207)
Kontomire leaf	3.483(±0.070)	5.583(±0.321)	3.660(±0.296)
Tomato fruit	1.883(±0.082)	2.213(±0.160)	3.710(±0.046)

C2 is the control farm. Maximum levels (MLs) of Pb in leafy and fruiting vegetables according FAO/WHO (CODEX Alimentarius Committee, 2011) are 0.2 mgkg^{-1} and 0.05 mgkg^{-1} respectively. Averaged Cd concentration in soil is 0.420mgkg^{-1} ±SEM.

consumption. The tomato fruit on the other hand recorded about 38 to 74 times the maximum amount of Pb in fruits allowed for consumption (0.05 mgkg^{-1}) (Table 4 and Figure 4). Similar to the trends of Cd accumulation, the tomato leaves contained about twice the amount of Pb in the

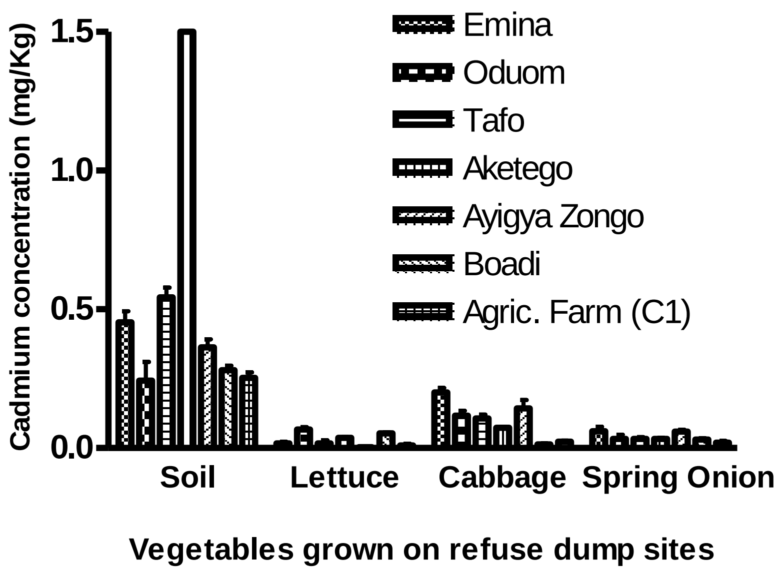

Figure 1. Cadmium concentration (mgkg^{-1}) in soil and vegetable samples from six refuse dump sites in the Kumasi Metropolis and a control site (Agric farm at Ayeduase) (N = 9, bar = standard error of mean).

tomato fruits (Table 4).

DISCUSSION

In the past decade, Ghana's vegetable production, local consumption and export have recorded between 20 to 25% growth annually (Sangare et al., 2012). This phenomenon is partly attributed to the increasing promotion of vegetable consumption among the local population due to its health benefits and the ever increasing horticultural export industry to countries such as those in the European Union (Thow and Priyadarshi, 2013). Despite the health benefits associated with the consumption of vegetables, a number of diseases have been linked to it (Reiss et al., 2012). Prominent among these vegetable associated diseases are those caused by microbial pathogens and toxigenic chemical compounds (Singh et al., 2011b). Dumpsite agricultural lands contribute to vegetable production especially in the urban communities where arable lands are scarce (Dubbeling and De Zeeuw, 2011). However, these dumpsite used for agriculture are important sources of dangerous heavy metals derived from components of industrial products (Fuge, 2013; Wuana and Okieimen, 2011) and thus agricultural activities on such lands provide entry route for heavy metals in the food chain.

All the eight dumpsites studied were used in the cultivation of various vegetables for human consumption. The levels of Cd and Pb, the two most injurious heavy metals to human health (Islam et al., 2007), in soil samples from the dumpsites were found to be higher than dosages approved safe for agricultural activities especially crop farming (Figures 1 to 4).

The waste composition of the dumpsites as well as the state of decomposition (or the age of the established dumpsite) affected the levels of heavy metals in the soil. Aketego dumpsite recorded 13.62 mgkg^{-1} Cd in soil, a level several times over the amounts allowed for agricultural purpose as recommended by the FAO and EPA (Bay, 2013). Pb levels in the soils from all the dumpsites were exceptionally high above the levels approved for agricultural activity (Figures 3 and 4). The results clearly show that the soils at the dumpsites may not be suitable for agricultural activity especially for growing crops such as those assessed in this study. Alternatively, these fields could be decontaminated of heavy metals through programmes involving plants with high bioremediative power for Pb and Cd.

Industrial waste is the most important sources of Pb exposure. Pb element and its compounds are components of a number of industrial products such as batteries, cables, pigments, plumbing and ceramics, gasoline, tobacco, solder and steel products, food packaging glassware and pesticides (Tangahu et al., 2011). Naturally, Cd is rare in the environment but its levels in agricultural fields have risen as a result of human activities (Cranor, 2011). Like Pb, the major source of Cd is the disposal of industrial waste containing Cd. Cd is used in the production of batteries, coatings, stabilizers and pigments (Faroon et al., 2012). These industrial wastes are dumped in Kumasi and other cities in Ghana by importers of used electronic products and machine parts from the Unites States, Europe and Asia (Schaller et al., 2009).

The leafy vegetables (lettuce, cabbage, spring onion,

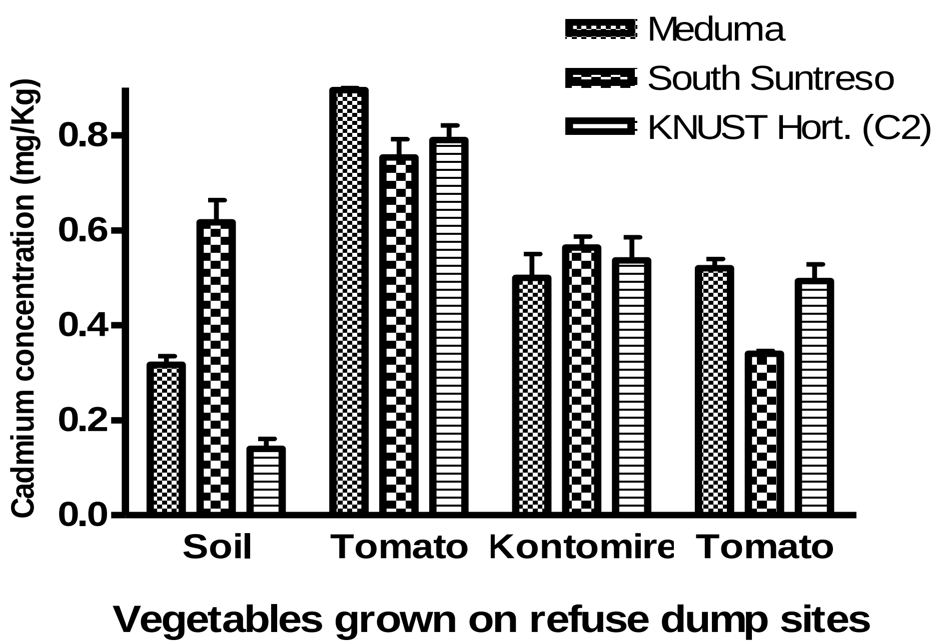

Figure 2. Cadmium concentration (mgkg^{-1}) in soil and vegetable samples from six refuse dump sites in the Kumasi Metropolis and a control site (KNUST Horticulture) (N = 9, bar = standard error of mean).

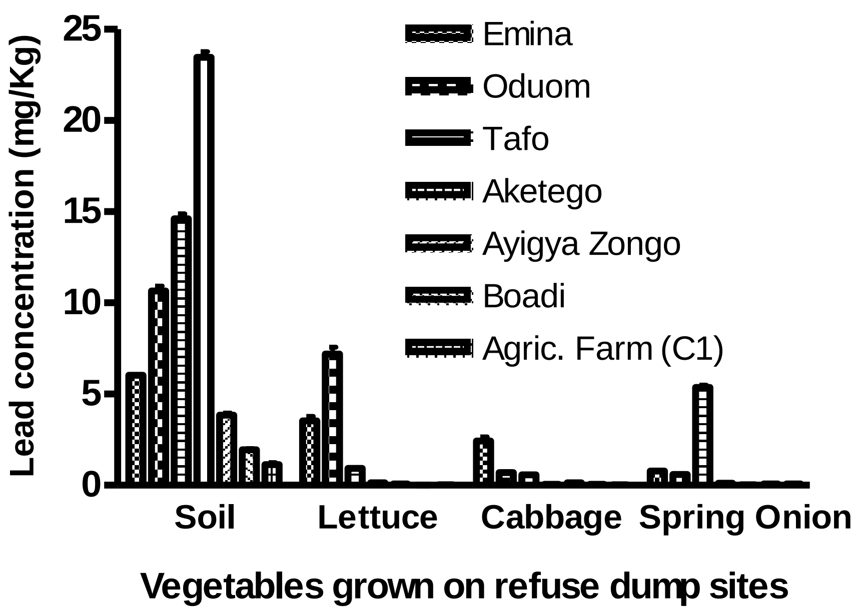

Figure 3. Pb concentration (mgkg^{-1}) in soil and vegetable samples from refuse six dump sites and a control site (Agric Farm at Ayeduase) in the Kumasi Metropolis of Ghana (N = 9, bar = standard error of mean).

kontomire and tomato leaves) growing in soils with high levels of Cd and Pb accumulated the heavy metals at levels far above the maximum concentrations approved by the WHO and FAO for human consumption. Naturally, leafy vegetables accumulate higher levels of Cd and Pb than other crops (Mench, 1998). The uptake of Cd by plants and fungi from the soil is directly related to the concentration in the soil (WHO/FAO, 2011; Ling-Zhi et al., 2011). Generally, Cd uptake by plants from the soil is enhanced by low soil pH (Rajkumar et al., 2012), a

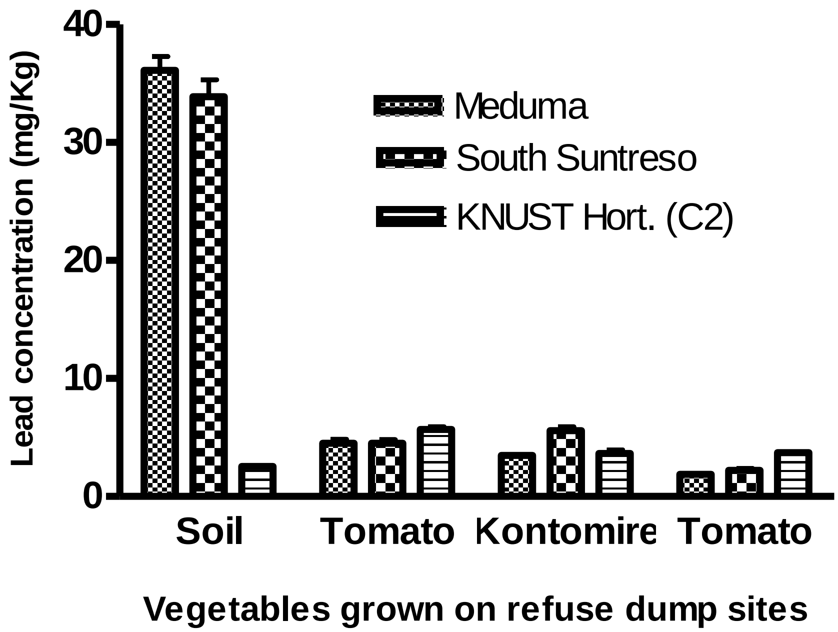

Figure 4. Pb concentration (mgkg^{-1}) in soil and vegetable samples from refuse dump sites in the Kumasi Metropolis of Ghana (N = 9, bar = standard error of mean).

feature commonly associated with industrial acidic waste. It was observed in this study that vegetables from the KNUST Horticulture, one of the control agricultural farms, produced vegetables with Pb and Cd levels significantly above the recommended concentrations for human consumption (Tables 2 and 4). Although, no anthropological data on the site is available, discussions with indigenes living around the university indicate that some parts of the KNUST agricultural fields were once human settlement and thus could contribute to the high metal contents of the soil (personal interviews).

Consumption of these vegetables cultivated on these dumpsites in the Kumasi Metropolis contaminated with high levels of Pb and Cd poses serious health risk to consumers. Pb is considered a classical chronic or cumulative poison. In humans, Pb can result in a wide range of effects including hematological effects, neurological and behavioral effects, renal effects, cardiovascular effects, and effects on the reproductive system (WHO/FAO, 2011; Abdullahi, 2013; Patra et al., 2011; Sun et al., 2014). Children are more vulnerable to effects of Pb than adults. Pb has been shown to be associated with impaired neurobehavioral functioning in children and this remains the most critical effect of Pb (Grandjean and Landrigan, 2014).

Based on dose-response analyses, it has been established that provisional tolerable weekly intake (PTWI) of 25000 mgkg^{-1} body weight is associated with a decrease of at least 3 IQ points in children and an increase in systolic blood pressure of approximately 3 mmHg (0.4 kPa) in adults (WHO/FAO, 2011; Solenkova et al., 2014; Tellez-Plaza et al., 2012). The rate of absorption of Pb into the bloodstream ranges from 3 to 80% (WHO/FAO, 2011; Marsh and Bailey, 2013; Sabath and Robles-Osorio, 2012). Younger persons and fasting individuals tend to absorb higher amounts of Pb ingested or inhaled. Half-life of Pb in blood and soft tissues is about 28 to 36 days (Farzin et al., 2008). Cd excretion is very slow and thus having very long half-life (decades) once in the body (WHO/FAO, 2011; Edwards et al., 2009). The major health risk associated with Cd exposure in renal failure due to high accumulation in the kidney exposure (Kazi et al., 2008).

In conclusion, soils from the eight dumpsites sampled from the Kumasi Metropolis recorded very high concentrations of Pb and Cd and these metals were accumulated in the tissues of vegetables cultivated on them. The levels of Cd and Pb in the vegetables exceeded the maximum levels (ML) approved by WHO for human consumption. Therefore, consumption of vegetables cultivated on these dumpsites poses serious health risk to the consumers.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Eucalyptus urocan drought tolerance mechanisms

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The present study was designed to identify strategies for tolerance to hydric deficit in *Eucalyptus urocan* seedlings. The experiment was conducted in a green house with 100% solar radiation capture. The completely randomized block design was used with five treatments (plants irrigated daily with water corresponding to 25, 50, 75, 100 and 125% daily evapotranspiration) and five replications. 120-day-old *E. urocan* seedlings (hybrid result from the crossing between *Eucalyptus Urophylla* x *Eucalyptus camaldulensis*) were planted in pots containing 8 L of substrate composed by oxisol, sand and muck at 3:1:0.5 ratio, respectively. The seedlings were irrigated with different volumes of water for 13 days and then analyzed. Under hydric deficit condition, *E. urocan* plants showed significant investment in the root system, reduced the breathing rate and kept enough turgor for growth. *E. urocan* plants at initial growth stage are tolerant to hydric deficit and show dehydration delay as a strategy to tolerate drought.

Key words: Hydric deficit, silviculture, initial growth.

INTRODUCTION

Brazilian forestry sector accounts for 24% of the agricultural GDP and 1.2% of all the wealth generated in the country. Brazil holds the most advanced technologies for the exploration of eucalypt and achieves yields higher than the species origin pole, Australia. Despite the successful performance in the sector, planted forests take less than 1% of the country's productive area and show great growth potential from commercial exploration of new areas (Ibge, 2014).

The Eucalyptus is the most cultivated tree genre in Brazil, which represents about 60% of commercial plantations. The Eucalyptus genus species found in Brazil suitable soil and climate conditions for rapid and appropriate growth in a short period of time. Its large-scale use is fostered by multiple applications, as "from eucalypt everything is usable". Essential oil extracted from the leaves is used in food, cleaning products, perfume and even in medicine. The bark provides tannin, and the trunk, posts, poles, mine props, boat masts, packaging boards and furniture. The fiber is used as raw material for papermaking and cellulose (Abraf, 2013). Despite the success of planted forests, the sector may

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generate even more wealth if it explores areas unsuitable for several other crops less tolerant or sensitive to abiotic stresses (Lopes et al., 2015).

Valuation of cropping areas and reduction of available ones have pushed eucalypt away to marginal areas, such as regions with low water availability and low fertility soils. Amongst all environmental resources required for agricultural productivity, water is the most abundant and also the most limiting one. Water shortage is the greatest barrier to agricultural productivity. Approximately 35% of the Earth's land is arid or semi-arid, and does not get adequate rainfall water supply for most species grown (Diaz-López et al., 2012). Current forecasts point to continuing global warming and increasing periods of drought in many regions of the planet. During drought, these plants are subjected to water shortage, which significantly hinders the accumulation of biomass. Water deficit reduces metabolic capacity and alters exchange activity with significant damage to wood formation and anatomy (Sette Jr. et al., 2010).

During the initial development phase, water deficit decisively affects growth and increases tree mortality in the field (Cabral et al., 2010). The intensity of the symptoms and the disorders observed during the drought are important variables for identification of promising materials for semi-arid regions (Reis, 2011). Drought tolerance is a result of several features that express themselves in different manners and at the same time, depending on the severity and frequency of the water deficit, the plant age and nutritional conditions, type and depth of soil, and atmospheric evaporative demand. Therefore, the adoption of a single strategy for drought adaptation is certainly not enough in any kind of environment (Sambatti and Caylor, 2007).

The conducting of research to identify drought tolerance mechanisms in eucalypt plants can increase the species productivity in traditional growing regions that have started to experience sporadic droughts and maximize commercial exploitation through the introduction of the species in areas previously considered unsuitable. This study aimed to identify the strategies for water deficit tolerance in *Eucalyptus urocan* plants.

MATERIALS AND METHODS

Experimental design

The work was carried out on a bench with transparent covering at Goiás State University, Ipameri Campus (17°43'19''S, 48°09'35''W, 773m alt.), Ipameri, Goiás. This area has AW weather according to Köppen classification. During the experiment the average relative humidity was 46%, and maximum and minimum average temperatures were 31 and 14°C, respectively.

The experiment was carried out following the completely randomized design, with five treatments (plants irrigated daily with water volumes corresponding to 25, 50, 75, 100 and 125% of daily evapotranspiration), five replicates and parcel of a plant in a pot. The water volume referring to daily evapotranspiration was calculated according to recommendations by Allen et al. (1998). 120-day-old *E. urocan* seedlings (originated from a hybrid cross between *Eucalyptus Urophylla* x *Eucalyptus Camaldulensis*) were planted in 8-L pots with substrate composed of oxisol, sand and manure at 3:1:0.5 ratio. Chemical analysis of the mixture showed the following values: pH 6.4; 19 g dm^{-3} OM; 2.4 mg dm^{-3} P; 109 $cmol_c$ dm^{-3} K; 1.5 $cmol_c$ dm^{-3} H+Al; 3.2 $cmol_c$ dm^{-3} Ca; 1.6 $cmol_c$ dm^{-3} Mg; 27.7mg dm^{-3} Zn; 77.20% SB and 6.58 CTC. After the composition analysis, decision was made to not do fertilization, nor correct the substrate pH. Seedlings were irrigated with different water volumes for 13 days, and then the following variables were analyzed: number of leaves, leaf length and width, plant height, stem diameter, relative water content (RWC), chlorophylls and carotenoids, root mass ratio (RMR), stem mass ratio (SMR), leaf mass ratio (LMR), transpiration and total biomass.

Growth variables

The plant height, leaf length and width and stem diameter were measured by using a graduated ruler and a digital caliper. The number of leaves was obtained by counting all the plant leaves. The destructive analysis was then performed, when leaves, roots and stems were separated and oven-dried at 72°C to reach constant dry matter, and then weighed. The leaf mass ratio (LMR), root mass ratio (RMR), stem mass ratio (SMC) and total biomass were calculated based on the dry mass data.

Relative water content in the leaf (RWC)

In order to obtain the relative water content, five leaf discs were extracted each repetition of 6 mm diameter, weighed and saturated in Petri dishes with distilled water for 24 h. Then the discs were weighed again and dried at 70°C for 72 h for subsequent recording of the dry weight in grams.

Transpiration

Daily transpiration was estimated gravimetrically, by determining the difference in weight of the pots as described by Cavatte et al. (2012). However, a 24 h time interval was used with weighing at 6 pm.

Photosynthetic pigments

To determine the total chlorophyll and carotenoid concentration, foliar discs were extracted from a well-known area and put into glass containers with dimethyl sulfoxide (DMSO). Later, extraction was performed in water bath at 65°C for one hour. Aliquots were taken for spectrophotometric reading at 480, 649.1 and 665 nm. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid contents were determined according to the equation proposed by Wellburn (1994).

Data analysis

The data were submitted to variance analysis and then to regression analysis using the R software (R Core Team, 2015).

RESULTS

Analysis of variance showed no significant change in mass ratio stem, number of leaves, plant height, leaf concentrations of carotenoids and chlorophylls (a + b) by

Table 1. Analysis of variance and regression equations for relative water content (RWC), biomass (BIO), root mass ratio (RMR), height, mass ratio stem (RMS), leaf mass ratio (RMF), transpiration (*E*) in plant eucalypt under water deficit.

Variables	Mean squares		
	Regression	Residual	Equations
GF	1	23	--
RWC	5873.9**	117.8	$Y = 83.1(1\text{--}e^{-0,03x})$; $R^2 = 0,70$
BIO	70.7*	11.7	$Y = 10.93+0.05x$; $R^2 = 0.95$
RMR	0,02**	0,01	$Y = 0.39-0.001x$; $R^2 = 0.97$
Height	28.8 ns	39.05	There was no adjustment equation
RMS	0,005 ns	0,003	There was no adjustment equation
RMF	0,10**	0,008	$Y = 0.32+0.002x$; $R^2 = 0.97$
E	202884**	865.3	$Y = -44.5+2.548x$; $R^2 = 0.96$

*significant at 5% probability; **significant at 1% probability; ns = non-significant by F test.

Table 2. Analysis of variance and regression equations for leaf width, leaf lengh, number of leaves, stem diameter, carotenoids (CAR), chlorophyll *a*, total chlorophyll Chl (*a*+*b*) and ratio of chlorophyll *a* chlorophyll *b* (Chl *a* / Chl *b*) in plant Eucalypt under water deficit.

Variables	Mean squares		
	Regression	Residual	Equations
GF	1	23	--
Leaf width	2.46*	0.51	$Y = 4.88+0.009x$; $R^2 = 0,41$
Leaf length	13.4*	2.40	$Y = 12.95+0.02x$; $R^2 = 0.44$
Number leaf	180.5 ns	209.5	There was no adjustment equation
Stem diameter	7.61**	0.25	$Y = 4.85+0.02x$; $R^2 = 0.99$
CAR	0,03ns	0,08	There was no adjustment equation
Chl *a*	0.66*	0.12	$Y = 1.31+0.005x$; $R^2 = 0.48$
Chl *a* /Chl *b*	0.94 **	0,05	$Y = 1.87(1\text{--}e^{-0,05x})$; $R^2 = 0.50$
Chl (*a*+*b*)	1.93 ns	1.67	There was no adjustment equation

*significant at 5% probability; **significant at 1% probability; ns = non-significant by F test. The biomass, stem diameter, leaf mass ratio showed increasing values with increasing availability of water, however, the root mass ratio values decreased with increasing water availability (Figure 1).

F test and the data did not fit in any regression model significant at 5% probability, and significant variations in relative water content, biomass ratio of root and leaf mass, sweating, stem diameter, leaf length and width (Tables 1 and 2).

The biomass, stem diameter, leaf mass ratio showed increasing values with increasing availability of water, however, the root mass ratio values decreased with increasing water availability (Figure 1).

The leaf chlorophyll a, ratio chlorophylls a/b, length and width of the fully expanded leaf showed increasing amounts with increasing water availability (Figure 2).

The transpiration and relative water content showed increasing values with increasing availability of water (Figure 3).

DISCUSSION

Tolerance to water deficit is critical for the expansion of the agricultural frontier through exploration of previously unsuitable areas. Maintaining growth and preventing metabolic damage under low water availability in the soil and/or in the atmosphere is important indication of drought tolerance (Matos et al., 2014). Eucalyptus showed a significant decrease in growth variables when subjected to water deficit as discussed subsequently.

Water shortage interfered significantly with initial growth of eucalypt plants. The reduced values of biomass, stem diameter, leaf mass ratio, length and width leaf combined with the high root mass ratio values in plants irrigated with little water indicates the high sensitivity of eucalypt seedlings to variation in soil water level. Growing in low water volume (25 and 50%) is indicative of drought tolerance, because under this condition the eucalyptus plant allocated high percentage of biomass to the root system, exploring larger volume of soil and possibly absorbing more water to maintain the necessary turgor for shoot growth. The largest biomass

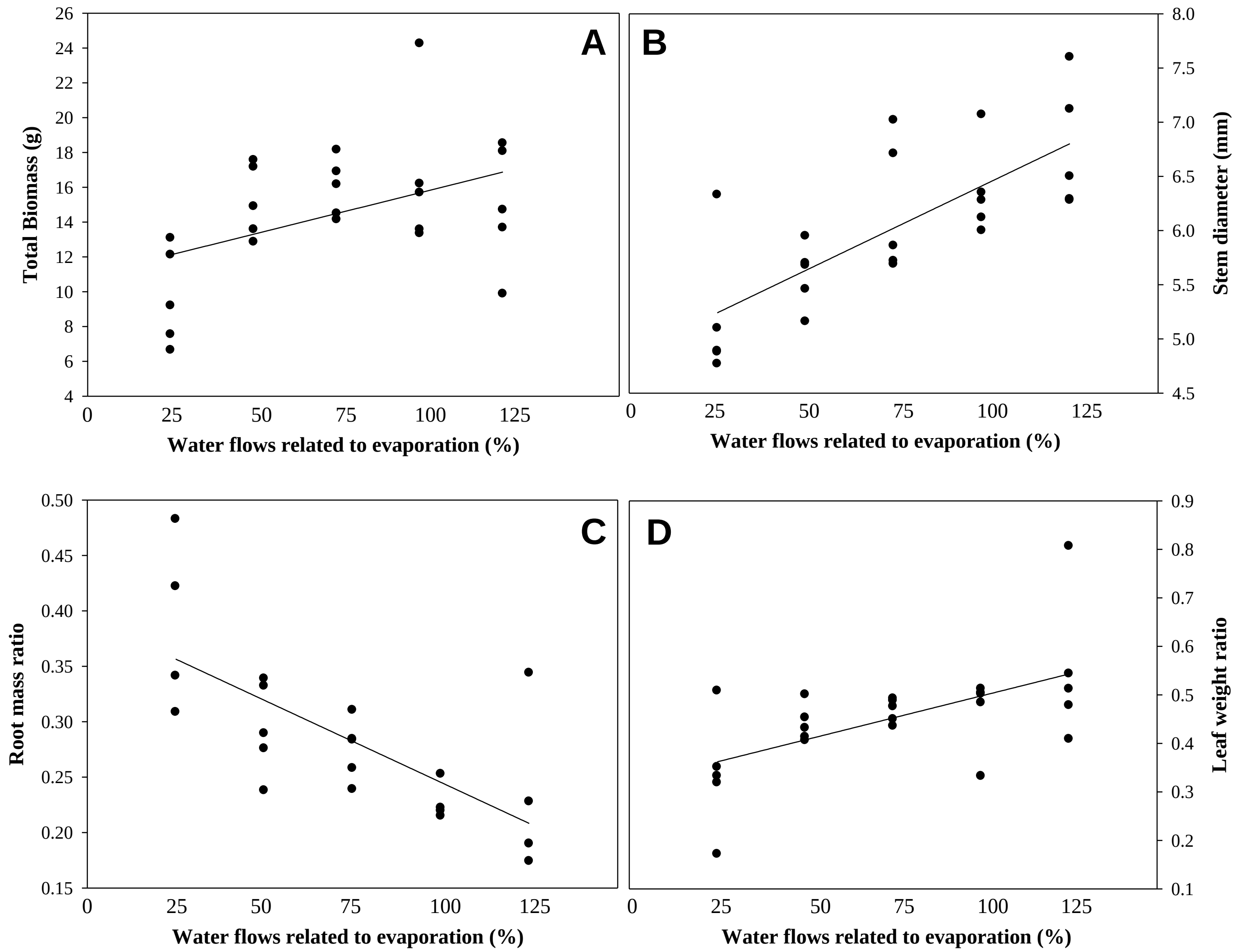

Figure 1. Regression equations for total biomass "A" [Y = 10.93+0.05x; R^2 = 0.95], stem diameter "B" [Y = 4.85 + 0.02x; R^2 = 0.99], root mass ratio "C" (Y = 0.39-0.001X; R^2 = 0.97) and leaf mass ratio "D" [Y = 0.32 + 0.002X; R^2 = 0.96] of *Eucalyptus urocan* seedlings irrigated with water volumes corresponding to 25, 50, 75, 100 and 125% evapotranspiration.

partitioning to the root system under reduced osmotic and water potential is a common response in several species (Góes et al., 2009; Matos et al., 2013; Souza et al., 2015.). Possibly, the eucalyptus plant has an efficient ground water extraction mechanism, for even under low water availability the plant draws water from the soil in sufficient volume to sustain growth.

The initiation and development of leaf primordia are dependent on the plant water status. According Taiz and Zaiger (2013), all of the absorbed water by the plant, 97% is transpired, 2% used in cell expansion and 1% in plant metabolism. Thus, low water availability reduced the leaf mass ratio. The lower biomass partitioning for leaves contributes to reducing perspiration and turgor maintenance. Among the morphological changes in water deficit condition, the reduced number and size of the leaves is the most significant (Santana et al., 2003; Souza et al., 2015; Diaz-López et al., 2012). Reduced transpiration in plants under water deficit may be associated with high stomatal sensitivity and lower "investment" in leaves. Low transpiration enabled maintenance or slight decrease in the relative water content in leaves and reasonable hydration sufficient for metabolism and growth. According to Souza et al. (2015), the reduced number of leaves, small change in relative water content and reduced plant transpiration in *E. urophylla* under low water availability is a sign of drought tolerance.

The non-significant variation in chlorophyll *b* concentration is indicative of absence of damage to D_1 protein of photosynthesis PSII and, thereby the variation in chlorophyll *a* / chlorophyll *b* ratio was due to changes

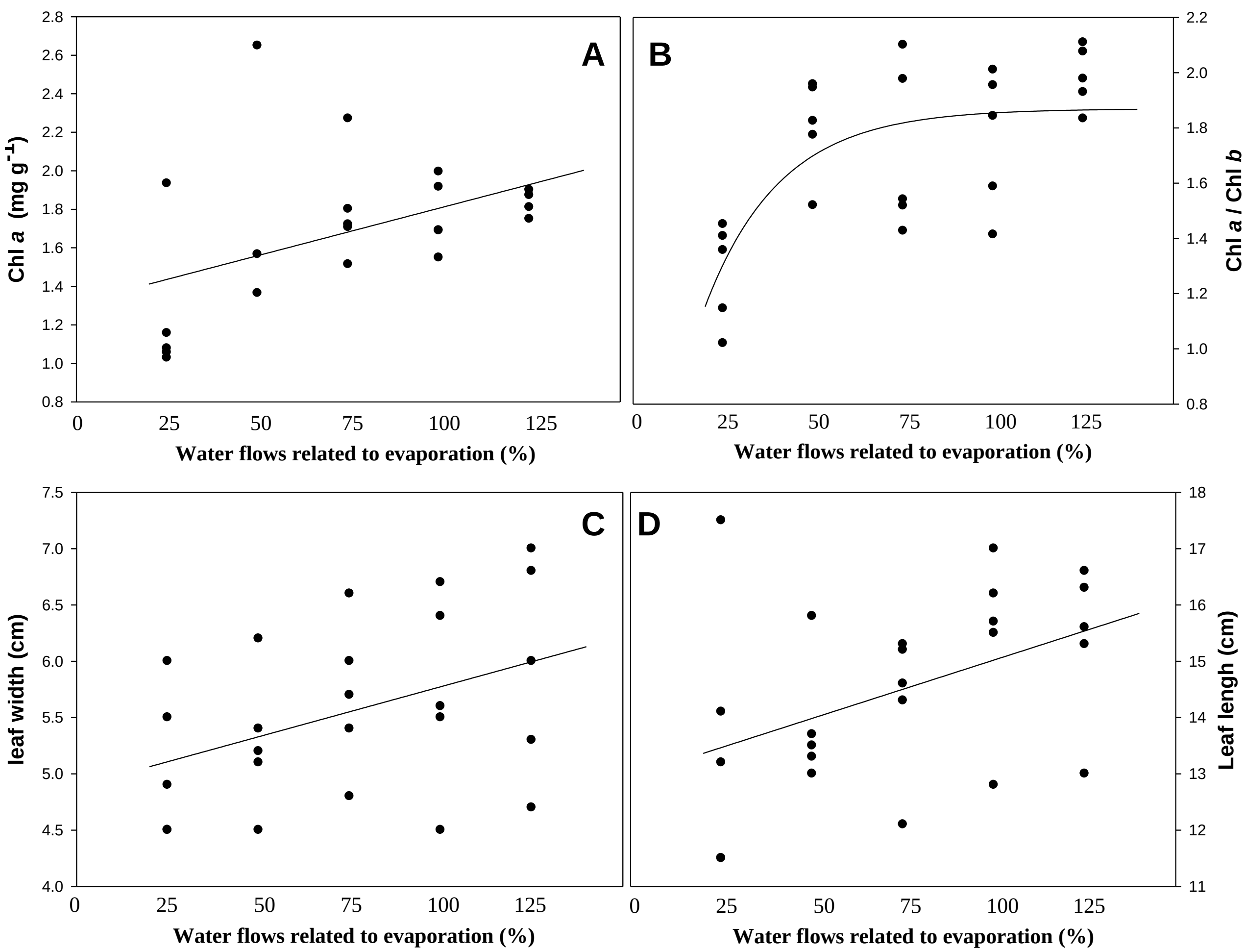

Figure 2. Regression equations for chlorophyll *a* "A" [$Y = 1.31 + 0.005x$; $R^2 = 0.48$], chlorophylls *a*/*b* ratio "B" [$Y = 1.87(1-e^{-0,05x})$; $R^2 = 0.50$] length leaf "C" [$Y= 12.95+0.02x$; $R^2 = 0.44$] and width leaf "D" [$Y =4.88+0.009x$; $R^2 = 0.41$] of *Eucalyptus urocan* seedlings irrigated with water volumes corresponding to 25, 50, 75, 100 and 125% evapotranspiration. Significant at *5 and **1% probabilities.

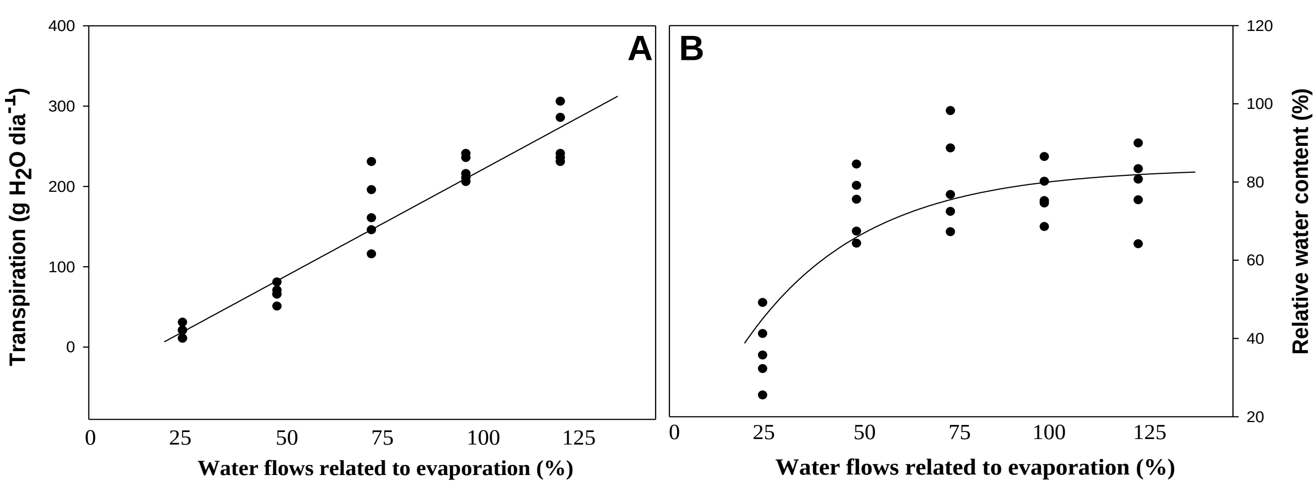

Figure 3. Regression equations for transpiration "A" [$Y = -44.5 + 2.548x$; $R^2 = 0.96^{**}$] and relative water content (RWC) "B" [$Y = 83.1(1-e^{-0,03x})$; $R^2 = 0.7^{**}$] of *Eucalyptus urocan* seedlings irrigated with water volumes corresponding to 25, 50, 75, 100 and 125% evapotranspiration. Significant at *5 and **1% probability.

in foliar concentration of chlorophyll *a* and may be associated to plant photoprotection against oxidative stress, since under low stomatal conductance, occurrence of excess excitation energy is common.

According to Matos et al. (2009), reduction in light energy absorption by lower leaf chlorophyll concentration is an important protective strategy against oxidative stress.

Under water deficit condition *E. urocan* plants showed significant investment in the root system, reduced transpiration rate and turgor maintenance sufficient to sustain growth.

Conclusions

1. *E. urocan* plants at initial growth stage are tolerant to water deficit, but shows a different biomass partition.
2. *E. urocan* plants use delayed dehydration as a strategy to tolerate drought.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Growth of *Khaya senegalensis* plant under water deficit

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The objective of the present study was to evaluate the initial growth of *Khaya senegalensis* plants under water deficit. The work was carried out at Ipameri, Goiás on a bench in full sun following the completely randomized experimental design with six treatments and six replications. 120-day-old mahogany plants (*Khaya senegalensis*) grown in eight-liter pots were subjected to six treatments for 12 days (plants irrigated daily with 100, 80, 60, 40, 20 and 0% of evapotranspiration) with six replications. At 132 days after emergence, the plants were assessed for: plant height, stem diameter, number of leaves, foliar area, daily transpiration, relative water content, total chlorophylls and carotenoids, leaf, stem and root mass ratios, and total biomass. The data were submitted to F-test and, when significant, to regression test at 5% probability. High stomatal control, reduced transpiration, low leaf concentration of total chlorophylls and increased root system growth to the detriment of the shoot growth indicate that *Khaya senegalensis* is tolerant to moderate water deficit.

Key words: Silviculture, wood noble, forest physiology.

INTRODUCTION

Planted trees have high potential for generating wealth in Brazil. The competitiveness of the Brazilian forestry sector, resulting from technological development and fast growth and adaptation of forest species, places the country in a prominent position in the world market (Ferreira et al., 2012). The Brazilian forestry sector accounts for 3.5% of the gross domestic product (Abraf, 2013). Extensive forestry-suitable land associated with applied technology has increased the exploration of planted forests. Around 90% of the lumber produced in Brazil comes from planted forests and only 10% from plant extraction (Ibge, 2014). Despite the high potential for growth of the Brazilian forestry sector, the exploration of new areas depends on the tolerance of species to common abiotic stresses occurring in northeastern and mid-western Brazil.

Changes in climate have prolonged the frequency and intensity of dry periods and reduced rainfall in different

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regions of the world. Abiotic stress is the leading cause of low crop productivity worldwide, reducing the average yield of most crops by over 50%. In forests, drought is the major limitation to growth, establishment and survival of plants (Zang et al., 2014).

Dry periods are a challenge to plant growth and development as it causes significant metabolic changes. Low water availability decreases photosynthesis, stomatal conductance, transpiration rate and productivity of woody species. Under such circumstances, growth is typically restricted to the root system, as a strategy for water absorption from deeper soil layers (Kozlowski and Pallardy, 1997). Tolerance to water deficit is a result of several features that are expressed distinctly by the different species. Dehydration severity depends on plant age and nutrition conditions, soil type and depth, and atmospheric evaporation demand. Thus, the adoption of more than just one drought tolerance strategy is certainly suitable for any type of environment (Sambatti and Caylor, 2007; Taiz, 2013; Matos et al., 2014.).

Eucalyptus represents about 70% of planted forests in Brazil (Gonçalves et al., 2009). There is a need, therefore, to diversify the production of raw material by introducing promising drought hardy species for wood production such as *Khaya senegalensis*. The exploration of various species makes the sector less vulnerable to biotic and abiotic weather.

The African mahogany (*K. senegalensis*), an exotic species of the Meliaceae family, stands out for its excellent wood quality, high prices in domestic and international markets, wood appreciated for carpentry, woodwork, shipbuilding and production of decorative veneers (Nikiema and Pasternak, 2008). Its wood is considered to be hardwood with excellent commercial value and physical and mechanical properties similar to Brazilian mahogany (*Swietenia macrophylla*). Slow growth and high number of branches are undesirable characteristics of *K. senegalensis*. However, the timber commercial value and water deficit tolerance make the species promising to increase the agricultural frontier of forest species, particularly in areas unfit due to scarce rainfall (Pinheiro et al., 2011).

The *K. senegalensis* species evolved in a tropical wet and dry climate in West Africa (rainfall ranging between 600 to 800 annual mm) and most likely adapts well to semi-arid regions of Brazil. The *Khaya ivorensis* saplings tolerate short periods of moderate water deficit; however, this type of study is still limited to *K. senegalensis* (Albuquerque et al., 2013).

Information about *K. senegalensis* growth under abiotic stress condition is scarce and insufficient for the development of forestry programs. Elucidating physiological performance of *K. senegalensis* under water deficit condition is necessary for commercial exploration in arid and semiarid regions. Considering the need to seek information of this nature to enable the production of *K. senegalensis* in regions with low rainfall, as well as better understanding of the attributes of this species to tolerate drought, enabling its wide commercial exploration, this study aimed at evaluating the initial growth of *K. senegalensis* plants under water deficit.

MATERIALS AND METHODS

The work was carried out on a bench in full sun at the Goiás State University experimental unit in Ipameri Campus (17°43'19"S, 48°09'35"W, Alt. 773 m), Ipameri, Goiás. According to Köppen classification the region has tropical climate (Aw) with dry winter and rainy summer. Mahogany seeds (*K. senegalensis*) were sown in eight-liter pots containing a mixture of soil, sand and manure at 3:1:0.5 proportion, respectively for offering adequate water storage, aeration, mineral nutrients. After analysis of the mixture composition, the substrate pH correction and fertilization were made accordingly. At 120 days after germination the plants were submitted to six treatments for 12 days (plants irrigated daily with 100, 80, 60, 40, 20 and 0% of evapotranspiration) with six replications. The water volume supplied was estimated following recommendations of Allen et al. (2006). At 132 days after germination, the following variables were analyzed: plant height, stem diameter, number of leaves, leaf area, daily transpiration, relative water content, total chlorophylls and carotenoids, root, stem and leaf mass ratios, and total biomass.

Growth variables

The number of leaves, leaf area, plant height and stem diameter were measured using a graduated ruler and a digital pachymeter. Destructive analyses were then performed when leaves, roots and stems were oven-dried at 72°C until constant dry weight was reached, and then they were weighed separately. Using the dry matter data, the leaf, root and stem mass ratios and total biomass were calculated.

Transpiration

The daily transpiration was estimated by gravimetry, comparing the difference in weight of the pots at one-hour intervals from 07:00 and 18:00 according to Cavatte et al. (2012).

Leaf relative water content (RWC)

The relative water content was determined by extracting five 12-mm diameter foliar discs, which were weighed and saturated for 18 h in Petri dishes with distilled water. Subsequently, the discs were weighed again and dried at 70°C for 72 h, in order to obtain the dry matter weight. The specific leaf area (SLA) was obtained from the equation proposed by Pedó et al. (2013).

Photosynthetic pigments

In order to determine the total concentrations of chlorophylls and carotenoids (Chl *a*+*b*), foliar discs were extracted (third pair of fully expanded leaves) and placed in dishes containing dimethyl sulfoxide (DMSO). Subsequently, extraction was carried out in water bath at 65°C for three hours. Aliquots were extracted for spectrophotometric analysis at 490, 646 and 663 nm. Chlorophyll *a*

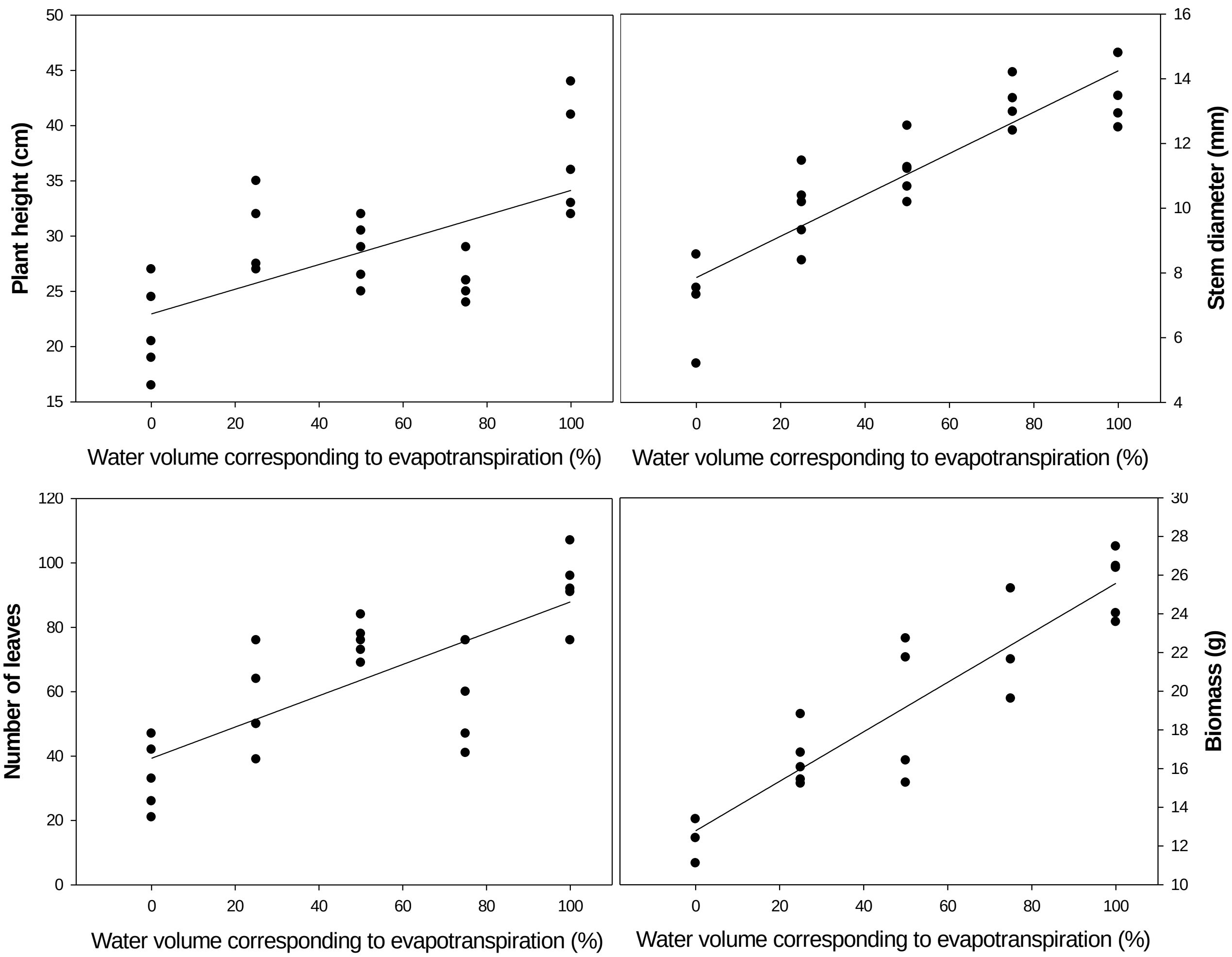

Figure 1. Regression equations for plant height Y=22.9604+0.1118x R2=0.97**, stem diameter Y=7.8564+0.0639x R2=0.99**, number of leaves Y=39.3200+0.4856x, R2=0.95** and biomass Y=12.7847+0.1279x R2=0.98** of Mahogany seedlings irrigated with different water volumes. ** Significant at 1% probability level by F test.

(Chl *a*) and chlorophyll *b* (Chl *b*) contents were determined through the equation proposed by Wellburn (1994).

Statistical procedures

Each water regime corresponded to one treatment. The variables were subjected to variance analysis following the completely randomized experimental design, with six treatments and six replications. The data were submitted to F-test and, when significant, to regression test at 5% probability. All statistical analyses were performed using SISVAR 5.3 software (Ferreira, 2011).

RESULTS

The regression curves for the growth variables: plant height, stem diameter, number of leaves and biomass are shown in Figure 1. All variables listed showed significant regression curve at 1% probability level for the F-test. The plants irrigated with water volume corresponding to 100% of daily evapotranspiration were 74% taller than the plants treated with 0% water. The same response pattern was observed for stem diameter, number of leaves and total biomass, so that the plants irrigated with 100% of evapotranspiration showed values higher than those of plants irrigated with smaller volumes of water (20, 40, 60, 80%).

The regression curves for relative water content, transpiration, leaf concentration of total chlorophylls and root mass ratio are shown in Figure 2. All the variables mentioned showed significant regression curve at 1% probability by F-test. The plants irrigated with 100% of the daily evapotranspiration water volume were 78% taller than the plants treated with 0% of water. The same response pattern was observed for the relative water content, so that the plants irrigated with 100% of

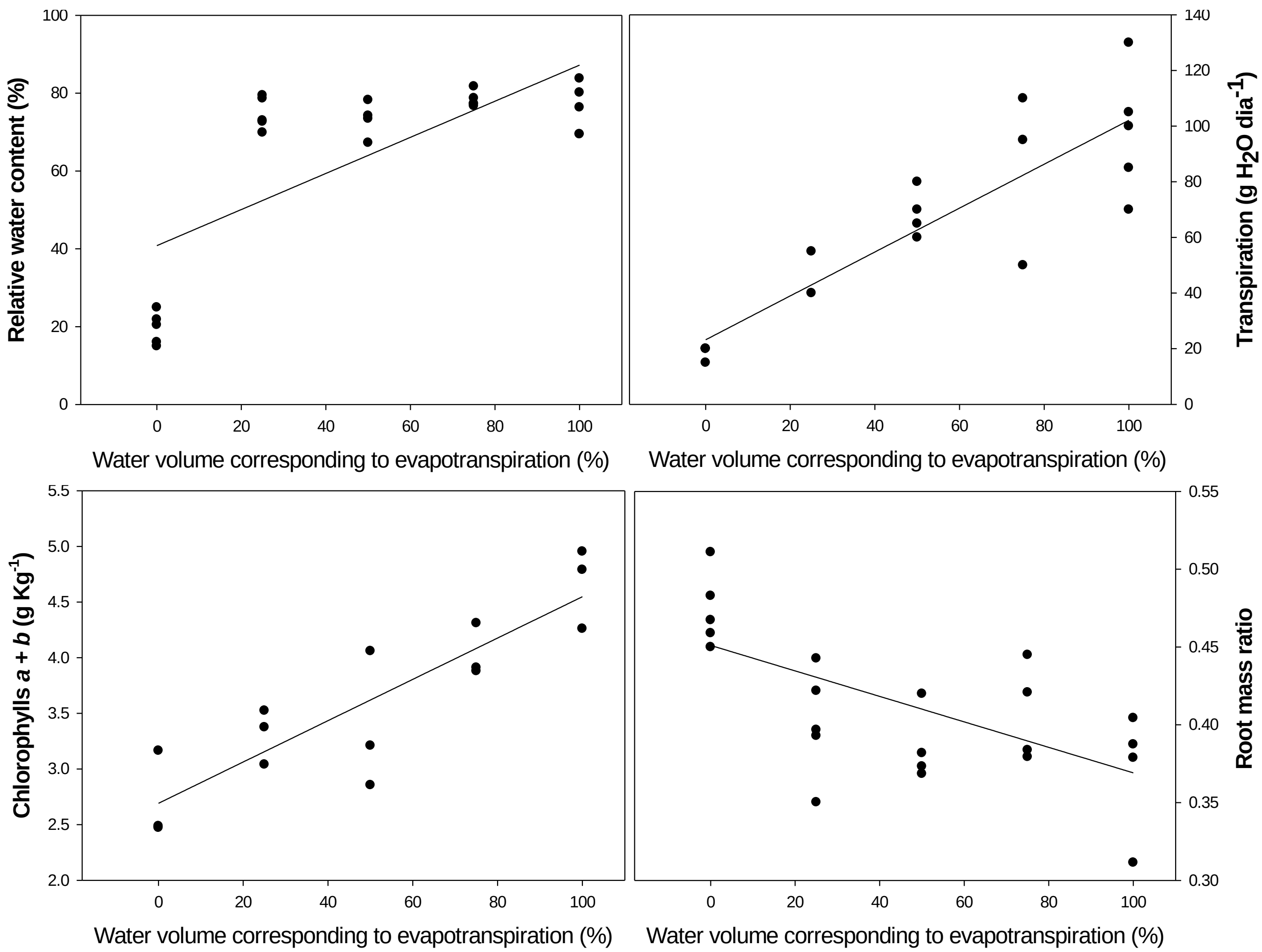

Figure 2. Regression equations for relative water content Y=40.8162+0.4639x, R^2=0.94**; transpiration Y=23.2084+0.7888x, R^2=0.78**; Y=2.6919+0.0186x, R^2=0.99**; chlorophylls (*a*+*b*) Y=2.6919+0.0186x, R^2=0.99** and root mass ratio Y=0.4510 – 0.0008x R^2=0.99** of Mahogany seedlings irrigated with different water volumes. ** Significant at 1% probability by F test.

evapotranspiration showed higher values than those of the plants irrigated with smaller water volumes (20, 40, 60, 80%). The leaf chlorophyll concentration was 59% higher in plants irrigated with 100% of evapotranspiration water volume. The plants irrigated with smaller volumes of water showed higher root mass ratio values. The plants treated with 0% of water content showed root mass ratio values 82% higher than those of plants irrigated with 100% of evapotranspiration.

DISCUSSION

The identification of forest species tolerant to drought is indispensable for the expansion of the agricultural frontier in the Brazilian semiarid region. Tolerance to abiotic stresses is a key factor for the survival and establishment of forest species in tropical ecosystems (Worbes et al., 2013). In forests, drought is a major obstacle to the establishment, growth and productivity of plants, as it is for most terrestrial plant communities (Allen et al., 2010; Luyssaert et al., 2010). Low water availability in the soil resulted in lower relative water content in the plant and considerably affected the growth of *K. senegalensis*. The reduced values of plant height, stem diameter and number of leaves in plants under water stress indicate that the species growth is highly sensitive to the plant water status. In addition, it is noted that plants irrigated with water volume equivalent to 80% of evapotranspiration already presented reduced variables. Studies have associated greater drought tolerance in trees with low growth rates (Rose et al., 2009; Taeger et al., 2013.).

The reduced transpiration rate in plants under water stress is associated with high stomatal sensitivity and efficient mechanism for water loss reduction through stomatal closing. The high stomatal control, typical of isohydric plants, has probably affected the carbon assimilation rate and, consequently, the accumulation of biomass and plant growth under low water availability, as stomatal closing also limits CO_2 inflow, while limiting the loss of water in vapor form. The results corroborate those found by Lima et al. (2007) when evaluating transpiration and stomatal conductance in *Swietenia macrophylla* plants under water deficit. According to Hommel et al. (2014), stomatal closing in response to water deficit alters the water use efficiency and decreases the photosynthetic rate.

The reduction in leaf chlorophyll concentration can be associated with the species photoprotection mechanism, because under water deficit condition, the formation of free radicals that damage membranes and proteins is common (Matos et al., 2009). In these circumstances the reduced absorption of light energy due to low chlorophyll concentration is an important morphophysiological adjustment to minimize the deleterious effects of excess photochemical energy. The high root mass ratio in plants under water stress indicates that even in a position of diffusive limitation and photosynthesis reduction, the plants partitioned assimilates to the root system growth. According to Albuquerque et al. (2013), *K. senegalensis* plants under water deficit invest part of the phosphate trioses in maintaining the starch content. Later on, glucose is broken, which through respiration produces ATP to support the growth of the root system. The results of this study corroborate those found by Ky-Dembele et al. (2010), who found greater root system growth when assessing growth responses of *K. senegalensis* under water deficit and Okali and Dodoo (1973) reported the reduction of perspiration and increase in the partitioning of assimilates to the root system of plants *K. senegalensis*.

The high stomatal control, low transpiration, reduced total leaf chlorophyll concentration and increased growth of the root system at the expense of the shoot growth show that *K. senegalensis* is tolerant to moderate water deficit.

Conclusions

1. *K. senegalensis* plants show efficient morpho-physiological adjustment to reduce water loss through transpiration and increase the root system growth under water deficit condition.
2. *K. senegalensis* plants are tolerant to moderate water deficit.

Conflict of Interests

The authors have not declared any conflict of interests.

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Characterization of family organic production in peri-urban regions

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The objective of this study was to evaluate the difficulties and potential of organic farming for the family small-scale farmers in the municipalities of metropolitan areas of large shopping centers, such as the state of Rio de Janeiro, Brazil. To that end, a questionnaire comprising open-ended and closed-ended questions relevant to biological farming was applied. Factors that hinder the production and sale of organic products were addressed. The data obtained using the questionnaire was compared with periodic technical visits to the farmers' properties. The results indicated that biological farming in the municipality is characterized as family-based, small-scale production and that products are mainly sold in farmers' markets. The group faces the following difficulties: A lack of continuous technical assistance, the limited availability of labor, and reduced consumer demand for the products. Farmers know and understand the importance of soil conservation processes in biological farming management and that this production system is based on health preservation, and needs to comprise affordable prices for the end consumer, and to expand the market.

Key words: Sustainable agriculture, family farming, organic products.

INTRODUCTION

Biological agriculture is defined as a production model that addresses socioeconomic and environmental issues. It combines livestock production, by using animal waste, and plant production, in addition to the use of residues such as rock powder and castor and sunflower cake originating from sites outside of the farm (Caldarte et al., 2012).

Biological agriculture is prominent because it is practiced worldwide and because it is a type of agriculture that prioritizes sustainable production, which has been found to be an important factor in social and environmental development. Agencies such as the Food and Agriculture Organization (FAO) evaluate the organic movement positively because it favors small-scale agriculture and

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because of the consequent revitalization of rural communities and environmental protection, especially in developing countries (El-Hage, 2007).

In Brazil, the incentive for the implementation of alternatives aimed at sustainable agriculture and agroecological production practices seeks to generate new perspectives so that a significant portion of the 13 million small farmers may insert themselves into the market because, until recently, this group has not benefited from public policies for rural development (Ferreira and Zannoni, 2001).

Studies show that the social and environmental benefits associated with biological farming, including environmental conservation and enhancement and stabilization of farmer income, positive impacts on local rural economy (Nieberg and Offermann, 2002; Lotter, 2003; Darnhofer, 2005).

Another important aspect addressed by Morgan and Murdoch (2000) refers to the participation of biological farmers' groups in all stages of the crop production process, from planning and implementation to commercialization, because the products are typically sold through direct relationships with consumers. However, part of biological farming remains tied to conventional sales channels that heavily dependent on financing capital, hindering the socioeconomic development of family farmers (De Wit and Verhoog, 2007).

At the state level, family farmers are represented by the Association of Organic Farmers of Rio de Janeiro (Associação de Agricultores Biológicos do Rio de Janeiro - ABIO), which is responsible for the participatory guarantee system throughout the state.

In this context, this article aims to analyze the development of urban agriculture in Brazil, studying the specific case of a group of organic farmers in the state of Rio de Janeiro. To do so, this article is structured in three topics: (a) a topic on a discussion on the characteristics of biological agriculture in Brazil; (b) a topic on the methods used and study area description; (c) a topic on a trajectory and development on the characteristics of organic agriculture in the municipality of Seropédica, located in the metropolitan area of Rio de Janeiro.

Biological agriculture in Brazil: Overview, advances, and the development of policies supporting organic agriculture

When biological agriculture began to be practiced in Brazil, it was closely linked to philosophical and social movements that sought the return of contact with the land as an alternative lifestyle. It was driven by a line of questioning that was against the consumerist model of modern society in the 1970s. During this same period, Brazil was already included as an organic product producing and consuming country in international statistics.

In the 1980s, several non-governmental organizations (NGOs) were founded to act in biological agriculture, coordinated through the Alternative Technologies Project Network (Rede Projeto Tecnologias Alternativas – PTA) and subsequently by the Advisory and Services, Alternative Agriculture Project (Assessoria e Serviços, Projeto Agricultura Alternativa – AS-PTA). However, growing interest in the organic export sector emerged, and thus, discussions of and debates over formulating and constructing Brazilian legislation for biological production began. Legislation was passed only after the global forum of NGOs and social movements that was held in Rio de Janeiro in 1992 - ECO 92 - and the approval of the 1994 European environmental legislation (Alves et al., 2012).

The issues concerning and negotiations for regulating biological agriculture in Brazil only occurred in 1994 and were officially recognized in May 1999 with the publication of the first Brazilian regulation on biological agriculture, the MAPA Regulatory Ruling 007/99 (Brasil, 1999). In 1996, the proceedings of the Brazilian National Congress began to pass Law 10,831, which defines and establishes mandatory conditions for the production and commercialization of biological agricultural products and which was only published in December 2003 (Brasil, 2003). In December 2007, Decree 6,323 was published, which regulates the activity, and in 2008, the specific regulatory rulings were published (Brasil, 2008a, b). Furthermore, the legal structure covers other regulatory rulings and decrees on the use of phytosanitary products and others subjects.

In August 2012, Decree no. 7,794 was approved, which implements the Brazilian National Policy on Agroecology and Organic Production (Política Nacional de Agroecologia e Produção Orgânica – PNAPO), establishing the Brazilian federal government's commitment to policies, programs, and actions that spur the agroecological transition and organic and biological production, collaborating with sustainable development and the improved quality of life of the population.

The scenario of biological agriculture in Brazil lacks information. The existing data are disseminated in the archives of farmers associations, certifying organizations, and NGOs and in the Agricultural and Livestock Census (IBGE, 2006).

Data from the International Federation of Organic Agriculture Movements (IFOAM, 2009) demonstrate that in 2008, a total area of 1,765,793 ha was organically farmed, including approximately certified 932,120 and 833,637 ha was in transition, with 7,250 farmers directly practicing biological agriculture.

The Agricultural and Livestock Census performed by the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística - IBGE) in 2006 recorded 90,497 established organic farmers in Brazil; 5,106 (5.6%) were certified, and 85,391 (94.4%) were uncertified. These data demonstrate the difficulty of farmers accessing certification, and in this case, it can be assumed that a considerable number of establishments are within a transition period. Of all of the establishments, 42% practice livestock production (meats, milk, eggs), and another 33% practice agriculture with temporary crops (soybean, corn, wheat, rice, bean, sugarcane).

Brazil has a diverse biological production that includes permanent crops (coffee, fruits, yerba mate), horticulture, floriculture (numerous species), forest production (planted and native), and extractive production and aquaculture.

Regarding the area of certified organic production, Brazil accounted for 4.9 million hectare, according to the last Agricultural and Livestock Census (IBGE, 2006); 4.4 million hectare were uncertified (89.5%), and 517,000 ha were certified (10.5%).

For funding and investing in agricultural production, the government currently provides two financing instruments: the national program for strengthening family agriculture (Pronaf) and the agricultural and livestock plan. Both financial aid lines offer support for biological and agroecological-based agriculture. There are also specific credit lines, including Pronaf Agroecologia, Pronaf Floresta, and Pronaf Eco. Female and young farmers also have distinct Pronaf lines, Pronaf Mulher and Pronaf Jovem (Brasil, 2014).

Other programs such as MAPA's Safra plan also have a credit line known as the Low-Carbon Agriculture Program (Programa de Agricultura de Baixo Carbono - ABC), which prioritizes the implementation and improvement of organic agricultural production systems, ABC Organic.

Within this scenario, organic agriculture began to be treated differently due to the grant for rural insurance starting with the 2012/2013 crop season. It is important to highlight that family agriculture insurance (seguro da agricultura familiar – SEAF) has ties to the Pronaf credit line, which is a specific policy for family agriculture, with multi-risk insurance covering losses due to adverse weather, fungal diseases, and/or pests without known or economically infeasible control techniques (CIAPO, 2013).

However, access to this insurance is hindered by both the low values covered and the restricted access to financing, especially due to the limited technical ability to develop projects and difficulty in gathering all of the information and documents necessary for requesting this financial support by some family farmers (Rocha and Santiago, 2013).

Biological farming in Rio de Janeiro

The biological agriculture movement in Rio de Janeiro state began in the early 1980s with a meeting of 400 people sharing a common goal: To produce and consume chemical contaminant-free products. The combination of this movement with the NGO "Harmonia Ambiental [Environmental Harmony]" resulted in the formation of Coonatura, which, in 1981, started ecological food production at a leased site in a district of Petrópolis, a mountainous region of Rio de Janeiro state (ABIO, 2014).

The group of farmers initially comprised individuals from urban areas who had a high educational level and who did not practice agriculture as their only source of income. The group's work focused on concerns with the quality of products sold to consumers and the impacts of contamination during farming (Campos, 2001).

This group's initiatives served as the basis for creating the first organic farmers association in the country. Thus, in 1985, the Association of Organic Farmers of Rio de Janeiro State (Associação de Produtores Biológicos do Estado do Rio de Janeiro - ABIO) was created, founded with the goal of disseminating biological agriculture and agroecology. ABIO supports organic farmers in their production and commercialization activities through cooperation and associativism and certifies the organic food produced by its members by supplying a certification seal (ABIO, 2014).

Feres (2009) highlights the importance of ABIO in encompassing most of the state's farmers and providing organic product certification, generating credibility for farmers and products, conferring the group greater transparency with regard to the practices and principles adopted for organic production in Rio de Janeiro.

Rio de Janeiro is not traditionally known for relevant agricultural production, and only some areas stand out, such as the northern Rio de Janeiro region in sugarcane production and the mountainous region in vegetable crop production (IBGE, 2014).

Nevertheless, Bicalho (2004) observes a change in the state's agricultural production that benefits the organic system, in which non-traditional and family farmers have adopted this farming technique, influenced by the ideological issues of the first biological farmers, the added value, and the increased demand for these products throughout Brazil. Thus, the state's organic production areas are practically located throughout all of its regions, covering areas without a history of agricultural activity and also sites near urban centers.

The development of biological agriculture in large metropolises is a reality, especially due to the strengthening of urban and peri-urban agriculture, thus ensuring self-sufficient food production and reduced dependence on the commercialization of products from other regions. Practicing urban agriculture has also allowed the families involved to strengthen communal activities, reducing the risks of food and nutritional insecurity and promoting increased income and employment for these areas (Weid, 2004).

To promote sustainable agricultural practices near urban centers, the ideal principles are agroecological principles, which involve crop rotation, alternative phytosanitary management, soil conservation and use, and the use of all of the available space and diversified production that help farmers obtain higher gains and allow selling throughout the year (Aquino and Assis, 2007)

Despite the uncertain biological agriculture market resulting from the lack of official data that identify production and the amount of capital generated by Rio de Janeiro and other municipalities involved in the production system, this market is currently booming, and the state has 319 farmers in the Brazilian National Register of Organic Farmers of the Brazilian Ministry of Agriculture and Federal Government (Cadastro Nacional de Produtores Orgânicos do Ministério da Agricultura e Governo Federal) (MAPA, 2014).

STUDY AREA AND METHODOLOGY

The study focus on the characterization of a group of farmer associated to SerOrgânico association, which represents biological farming in the municipality of Seropédica. SerOrgânico was founded in 2009 and currently has 15 registered farmers (Rede Ecológica Rio, 2014). To study the forms of agrobiological farming practiced by Seropédica farmers (registered in SerOrgânico), a survey was conducted combining questionnaire application and periodical technical visits to the farmers' properties in order to generate data on production management and its variables and consequently generate a production history.

The topics addressed were related to the agricultural difficulties faced by the farmers, the forms and alternatives found for commercialization, and the complexity of working with biological agriculture within the Seropédica region, following the procedures described in the current farmer’s manual published by the Brazilian Ministry of Agriculture, Livestock, and Supply (Ministério da Agricultura, Pecuária e Abastecimento – MAPA, 2011) related to the activities practiced at the farmers' production sites.

Although the municipality of Seropédica accounts for 15 units registered in the organic farmers association (SerOrgânico), only 11 continuously produce on their properties. Therefore, the research performed in this study was directed to these farmers due to their continuous production and commercialization over the years.

The study comprised visits to the farmers' properties and a questionnaire with open-ended and closed-ended questions aimed at agronomically evaluating the production system. Topics that are the main drawbacks related to the urban, family, and biological farming system in the municipality of Seropédica were addressed.

The questions were directed toward property size, the time needed to convert the conventional crop system to the biological system, and membership in ABIO. The following were also targeted in the study: the main soil conservation practices adopted, soil fertility management, pest and disease control, and invasive plants (Figure 2). Moreover, issues such as the source of the water used in irrigation, the commercialization of production, and the labor employed and concerns regarding the existence of continuous technical assistance were addressed.

The time that biological farming was performed in each property was based on the time of ABIO membership, given that this association issues the certificate of compliance to the producer.

Organic agriculture in Seropédica: Trajectory and development

Location and history

The municipality of Seropédica is located 70 km from the capital of the Rio of Janeiro (Figure 1) in a region geographically known as Baixada Fluminense and as a metropolitan region by the state's administrative political division (Ceperj, 2014). It encompasses an area of 283.8 km^2 and a population of 81,260 inhabitants, predominantly urban. The economy is based on sand and clay extraction and agriculture, which accounts for approximately 6,022 ha dedicated to family farming (IBGE, 2014).

The largest portion of these areas comes from the expropriation of unproductive farms that were managed by Brazilian federal agencies for agrarian colonization and reform in the 1950s. These expropriations led to nine settlements divided into lots, each averaging 10 ha. These centers produce fruit and vegetables but also practice more varied activities and differ in their degree of development (Golinski et al., 2007).

Despite the incentive to adopt the organic production system from research institutions such as the Federal Rural University of Rio de Janeiro (Universidade Federal Rural do Rio de Janeiro - UFRRJ), Embrapa Agrobiology (Embrapa Agrobiologia), and the Agricultural Research Company of Rio de Janeiro State (Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro - PESAGRO), which are located in this municipality (Cruz and Bigansolli, 2011), and ABIO itself, because of strong land speculation in recent years, many farmers have sold their properties, abandoned agriculture, or transferred their activities to other municipalities.

Biological family farming as alternative income for the municipality of Seropédica

Family farming is based on a production method in which the center of decisions, management, work, and capital is controlled by the family, whose income is sometimes supplemented by working wages. It is based on diversifying the products farmed to decrease costs, increase revenue, and take advantage of market opportunities and labor availability (Abramovay et al., 1996).

This farming method stands out because of its importance in generating employment and income and its collaboration in keeping rural space permanent, reducing rural exodus, and accounting for a considerable share of the national wealth generated. It is also responsible for food produced for domestic consumption, especially intended for self-consumption (subsistence farming), generating a source of resources for low-income families, thus significantly contributing to the economy of the national and regional agricultural sectors (Guilhoto et al., 2007).

According to data from the Brazilian Institute of Geography and Statistics (IBGE, 2006), 84% of the agricultural establishments in Brazil are family based, with 74% of individuals practicing some type of work in the field linked to family production and occupying 24.3% of the area used by Brazilian agricultural establishments. Therefore, this segment is indispensable for basic food production. Thus, understanding the factors that govern family farming in the municipality of Seropédica serves as an instrument for ensuring food security and the supply for local consumption, which may contribute considerably to the economy of this municipality and be an alternative for increasing income for the families involved in this production system.

Campanhola and Valarini (2001) emphasize biological agriculture as one of the income alternatives for small-scale farmers because of the increasing demand for healthier foods worldwide. Moreover, organic products exhibit characteristics of niche markets and thus aim to meet the demand of a restricted and select segment of consumers who are willing to pay a higher price for these products, which does not happen with *agricultural commodities*. Thus, although small-scale farmers do not achieve large-scale production, they can provide their products to small local markets, strengthening the trust and credibility relationships among the parties involved (Neto et al., 2010). Moreover, diversified planting and decreased dependence on amendments originating from outside the farm allow the income of organic family farmers to be stable throughout the year, reducing the effect of seasonality.

RESULTS AND DISCUSSION

Characterization of the biological production unit in the municipality

Biological farming is a recent agricultural method in the municipality, with respondents having on average 10 years of production certification, and therefore can be considered fragile, requiring care and attention.

Regarding the area intended for biological agriculture, it does not exceed 6 ha for any of the farmers interviewed. A portion of the area is reserved for pasture, either for owned or leased animal production. It is important to note that for half of the respondents, lease represents increased income (Figure 2). However, the area intended for biological production is always very diverse, which is important for practicing agroecologically based agriculture (Mesquita, 2013).

The group of farmers from Seropédica encounters the following main difficulties in developing agriculture in the municipality: a lack of continuous technical assistance, which generates a lack of current available information about crop management and difficulties in implementing the biological system (IBD, 2000). This factor differentiates this group of farmers from farmers in other regions of the state, such as those in the previously established green belt regions in the municipalities of Teresópolis-Nova Friburgo and Petrópolis (the mountainous region of Rio de Janeiro).

Interventions from educational and research institutions located in the municipality occur sporadically via courses, the dissemination and propagation of agronomic techniques, and partnerships aimed at installing and conducting experiments on the farms.

However, according to the farmers interviewed, there is no return from the results of the research conducted on their properties, which leads to skepticism with regard to the formation of new partnerships. A similar trend has been reported by Guimarães (2011), who evaluated the implementation of agroecological practices and found that only half of the family farmers evaluated (including conventional farmers) in Seropédica make use of agroecological practices, demonstrating that research and the consequent extension generated by educational institutions have not reached regional farmers in a relevant manner.

Among those interviewed in this study, 36% noted that the evaluation and interpretation of soil fertility is a predominant factor that contributes to increased production on the property and that the diffusion of this technique based on continuous technical assistance would bring benefits. It is noteworthy that practices such as no-tillage, crop rotation, and mulch are the main forms adopted for managing organic matter and soil moisture content, consequently improving the physical and biological characteristics of the system (Figure 2).

Regarding soil fertility management, liming is practiced in 65% of the farms surveyed. However, the procedure of interpretation and lime application is only performed correctly at two sites. Nitrogen is applied using castor cake, and phosphorus is applied using termophosphate. However, the uses of mulch, cattle manure, and biofertilizer are practices adopted by some of the organic farmers in Seropédica (Figure 2).

None of the respondents has mechanized agricultural tools to incorporate the fertilizers to improve soil fertility. However, it is common practice to rent this equipment during periods of soil preparation. The study found that all of the farmers know and understand the importance of soil conservation processes for soil management and its fertility for organic production. Thus, it can be affirmed that all of the farmers interviewed perform at least one agricultural practice with this goal.

Mazzoleni and Nogueira (2006) evaluated the organic production chain of a site close to Curitiba, Brazil, and compared certified and uncertified farmers, and they found that in 97% of the cases, the certified farmers performed practices that favor soil conservation and the environment, such as the use of mulch, no-tillage, and crop rotation.

Regarding pest or disease control, only 36% of the organic farmers in Seropédica use commercial products certified for use in organic agriculture; however, all use alternative control products such as solutions and extracts (Figure 2).

Similar to the findings of Malanski and Onçay (2011) in Campo Bonito, Paraná, Brazil, the biological farmers evaluated in the present study preserve the custom of seed sharing, work collectively, use popular knowledge to plant and harvest, observe the moon phases and the behavior of animals and birds, and make weather predictions. They believe in homemade phytopathological control comprising teas, syrups, and macerations.

Although there is a market interested in local biological production, none of the farmers demonstrated willingness to increase the area or plant a type of crop that they did not already produce. In all cases, labor was cited as the main limitation. According to Figure 1, the following main crops are produced by the group: annual crops, such as leafy vegetables, fruits, and medicinal plants.

Storch et al. (2004) also found such a situation when studying farmers from Rio Grande do Sul, Brazil. For these authors, labor was a recurring problem that was affecting various sectors of local production. It is noteworthy that the work force involved in the biological production of the SerOrgânico farmer association was family-based; however, 100% of the respondents hired day laborers for mowing and weeding (which were cited as main forms of invasive plant control). There was a migration of labor among the properties according to the demand for invasive plant control and soil management (Figure 2).

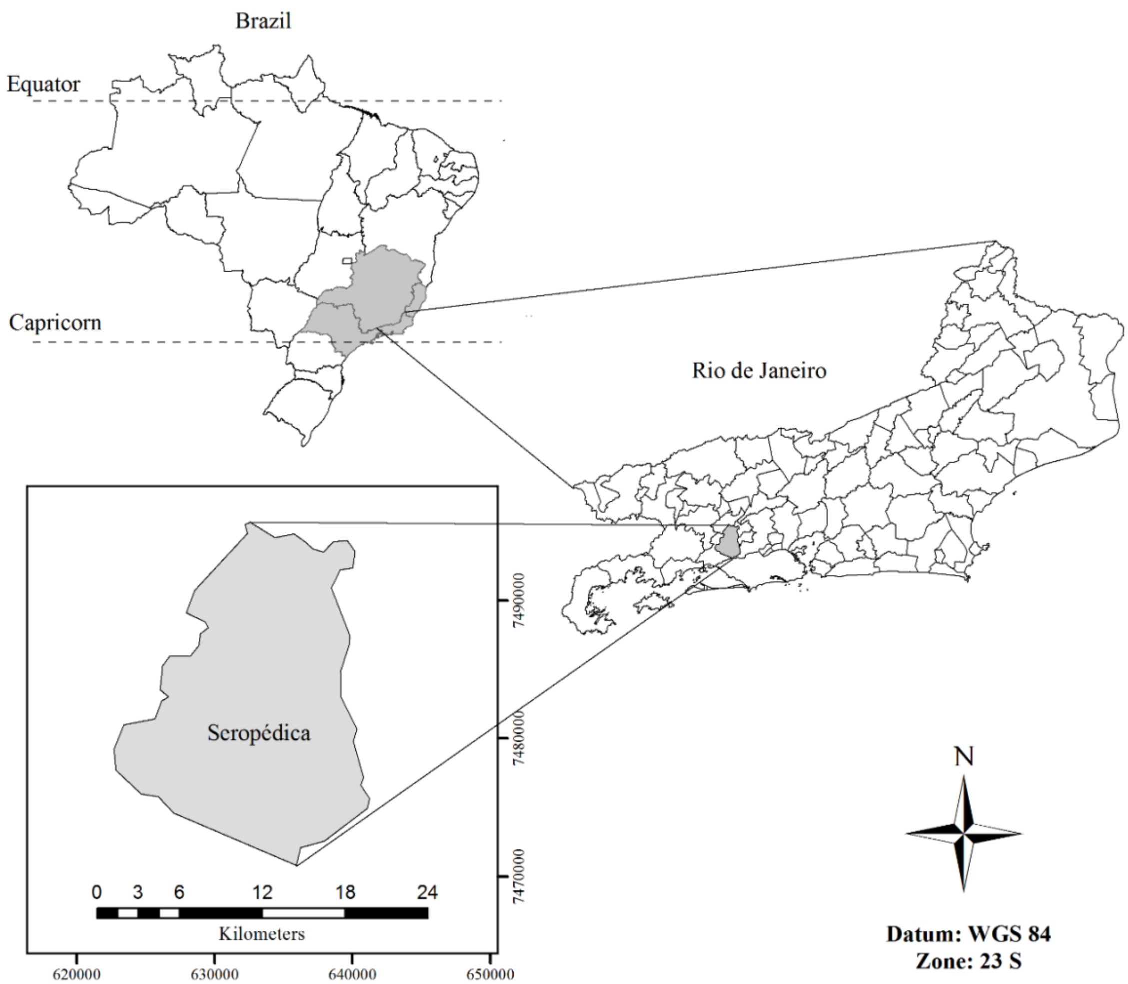

Figure 1. Location of the municipality of Seropédica.

Of the 11 respondents, six cited agricultural production as the main family income. The other farmers maintained an alternative occupation to increase the profitability generated by selling the production surplus.

Characterization of the agricultural production and commercialization in Seropédica- RJ

The main reason reported by biological farmers in Seropédica for adopting family-based and biological-based agriculture is health preservation, followed by price and market demand for the product.

Concern with the risks of using pesticides seemed to be widespread among the farmers who adopt the biological and agroecological production system, which was also reported as a factor of concern for farmers from Canada (Macrae, 1991), Rio Grande do Sul (Storch et al., 2004), and other regions of Rio de Janeiro (Aquino and Assis, 2007).

Due to climatic difficulties and low technological and financial investments, production in Seropédica is concentrated during two distinct periods: spring/summer and fall/winter. During the first season, the high temperatures in the municipality and the high disease rate do not allow planting leafy vegetables and medicinal plants by 100% of the respondents.

As an alternative, the farmers plant sweet potato, cassava, and corn and sell seasonal fruits during this period. During the cooler seasons, the number of products sold increases and includes cherry and table tomato, eggplant, and leafy and aromatic vegetables.

The products are sold in open farmers markets or via direct sale (Figure 2) belonging to the Organic Farmers Market Circuit of the City of Rio de Janeiro. Currently, all of the farmers sell their products in this manner. Moreover, 55% of the farmers sell their products to the Agroecology Network (Consumers Association) and the hotel chain within the state's Costa Verde region. In all cases, the respondents also sell their products directly to consumers who know the production site and go to the site itself (direct sale).

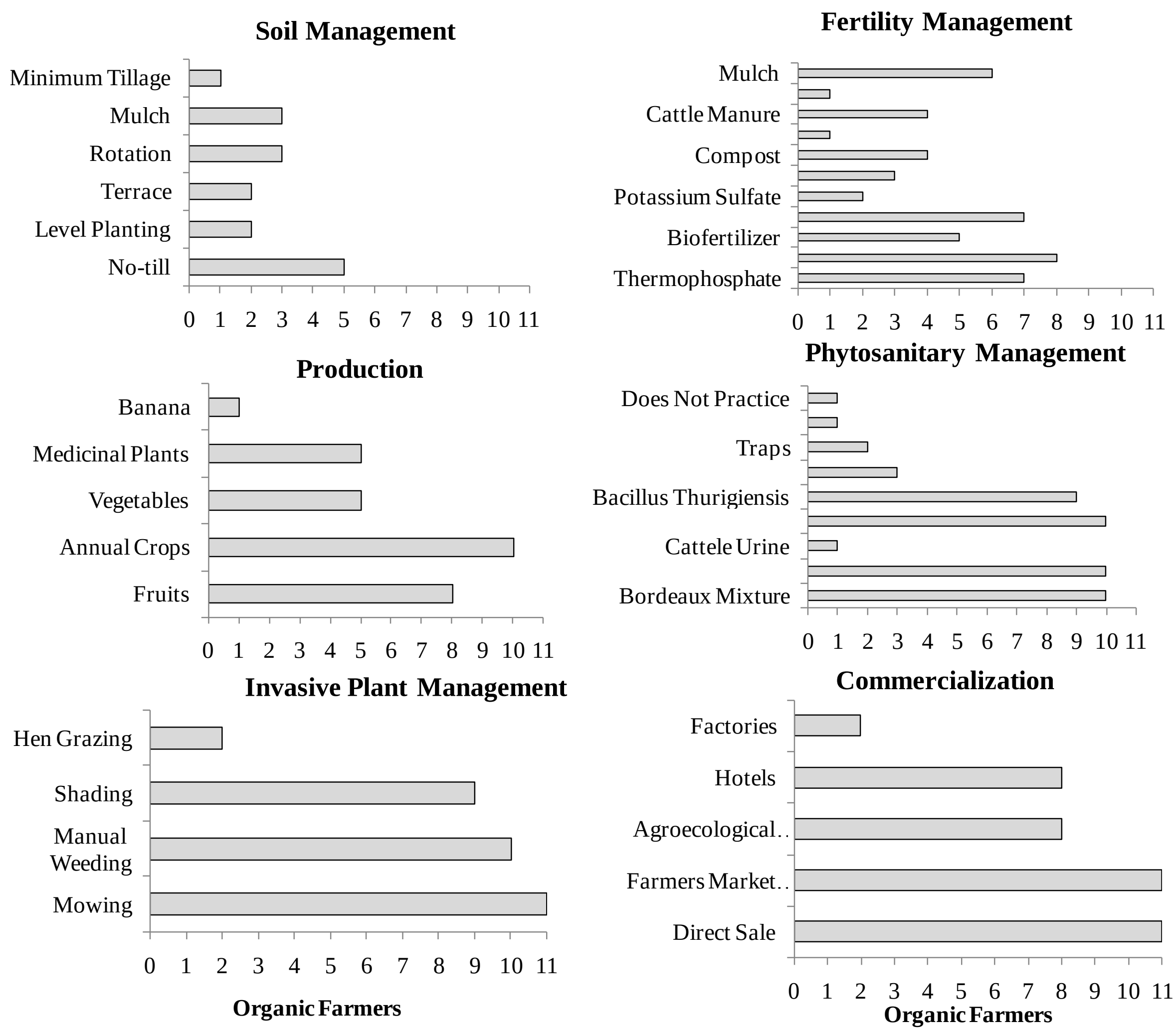

Figure 2. Types of organic production management adopted by family farmers in Seropédica, Rio de Janeiro.

According to Schultz et al. (2001), the agroecological production agents consider direct sales and sales in organic farmers markets to be the most appropriate methods for distributing their products, allowing farmers to be closer to consumers. However, this mechanism has limitations, mainly due to the possibility of increased demand that cannot be supplied and the requirement that the producers be present at these sites, reducing their ability to work at the production unit.

For Seropédica, this factor was cited as the main difficulty for selling at farmers markets, which occur Tuesdays, Thursdays, and Saturdays in different neighborhoods in Rio de Janeiro. The mean distance to these sites from the producing municipality was 70 km.

Conclusions

This study identifies some positive and negative factors of the biological production system adopted by farmers belonging to the SerOrgânico organic farmers association. Overall, the difficulties faced are similar to those of most small-scale farmers throughout Brazil. The positive aspects are as follows: the knowledge of conservation practices, application soil fertility improvement techniques, high product diversity, and effective phytosanitary control using appropriate amendments for the region's key pests and diseases. The negative aspects are as follows: a lack of continuous technical assistance, low production scale, irregular supply, which hinders long-term contracts with distributors and retail markets, and the limited availability of labor. Direct contact with consumers at commercialization sites favors trust in the products; however, the presence of the producers reduces their ability to work at the production unit. Another important aspect is the difficulty accessing specific credit lines and the lack of technical monitoring for production. The following are highlighted as positive aspects: Expanding the market for products, environmental preservation, the ability to diversify production, and the favorable prices of organic products.

Conflict of Interests

The authors have not declared any conflict of interests.

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Economic efficiency of green maize intercropped with beans grown under Tithonia and inorganic fertilizer

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A field trial aimed at assessing the performance of maize and beans under intercropped systems was conducted in 2013 under three experimental components of maize, beans and maize-bean intercrop in a randomized complete block design (RCBD) with three replications. Each component was subjected to treatments that included: a control-without fertilizer (WF), mineral fertilizer (MF) at 150 kg/ha of 17:17:17, Tithonia biomass (TDB) at 8 tons/ha and integrated application of MF and TDB comprising 4 tons/ha Tithonia and 100 kg/ha mineral fertilizer (MF/TDB). The MF/TDB produced 21.7% higher green maize yields than control, while MF and TDB produced 8.96 and 7.52% higher yields, respectively than the control. The land equivalent ratios (LER) were higher than one in all the intercropping plots, thus indicating an optimum exploitation of the environmental resources. Control plots showed the highest yield advantage in terms of LER of 1.70. When maize yield was converted to bean equivalent yield (BEY), the intercropping BEY was higher than the BEY in the sole for all fertilizer types, thereby revealing an agronomic advantage. The actual yield loss (AYL) values for maize indicated a yield gain of between 11.2 and 15.05% when MF and MFTDB were used in the intercropping compared to the sole cropping. Beans recorded yield loss in all the fertilizer types except in TDB which had a yield gain of 20.45%. The economic performance of the intercropping systems, affirmed that the most advantageous fertilizer type for maize was MF/TDB with an IA of 6.566. The monetary advantage index (MAI) indicated a definite yield and economic advantage in maize-bean intercrop over their sole cropping, with integrated use of MF/TDB as nutrient sources being the most economical and advantageous fertilizer regime.

Key words: Economic efficiency, land equivalent ratio (LER), green maize, beans, intercropping advantage, monetary advantage index (MAI), *Tithonia diversifolia.*

INTRODUCTION

Self-sufficiency in maize (*Zea mays* L) production is a major strategy for achieving food security in Kenya. The strategy is adopted to avoid undue reliance on unstable and unpredictable world food markets and to generate incomes to farmers and landless laborers (Mousavi and Eskandari, 2011). Apart from being grown for grain, maize can be produced ‘green’ to be consumed as a vegetable. Land in the high rainfall areas of Kenya is

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limiting due to high population; hence, it has become necessary to adopt intercropping as a way of increasing the land food output. Intercropping systems are more productive than sole crops grown on the same land, because they are associated with greater yield stability, greater land-use efficiency, increased competitive ability against weeds, improvement of soil fertility due to N fixation, and some favorable root exudates from leguminous species incorporated in the systems (Mousavi and Eskandari, 2011; Lithourgidis et al., 2006).

In Kenya, maize is among the crops that have been intensely grown in a mixture with other crops, especially legumes. Farmers prefer the legumes due to their inherent properties like short duration and ability to fix nitrogen. However, while most reports on intercrops have indicated increase in maize yields, Musambasi et al. (2012) reported a low maize yield when maize and cowpea (*Vigna unguiculata*) were intercropped and a high yield when maize and field beans *(Phaseolus vulgaris* L.) were intercropped.

The author associated higher intercropping yields with better utilization of growth resources, such as water, light and nutrients compared to sole cropping systems. Intercropping kale (*Brassica oleracea* var. *acephala*) and beans has shown increased productivity in terms of land equivalent ratios (LER). Intercropping soybean and maize gave LER values of 1.40 and 1.29, respectively indicating that higher productivity per unit area was achieved by growing the two crops together than growing them separately (Ijoyah et al., 2013).

Continuous and intensive use of highly priced synthetic fertilizer materials for boosting crop productivity in the past decades has been linked to rapid decline in tropical soil fertility and crop productivity. However, with much research efforts, the use of organic fertilizers with or without mineral fertilizers has been recommended to improve soil fertility and crop productivity. To this end, *Tithonia diversifolia* green biomass has been reported to be an effective source of nutrients and has been used successfully to improve soil fertility and crop yields in Kenya (Aguyoh et al., 2010). It has the ability to decompose and release nutrients rapidly. The integration of Tithonia biomass (TDB) with MF is consequently essential to supply sufficient nutrients in the soil. Synthetic fertilizers are expensive for majority of the peasant farmers, while green manuring is unpopular, especially where no edible crop is produced.

According to Gosh et al. (2006) imbalanced nutrient application coupled with low N and P content represent major constraints that limit crop productivity in intercropping systems in many soils where intensive cropping systems are practiced.

Osman at al. (2011) using monetary advantage index (MAI) reported significantly higher economic benefit when two rows of cowpea and one row of millet were intercropped compared to a mixture with one row of each of the crops. Although a number of field studies have been carried out to compare economics of the sole crop yield when taken along with other crops in the system, adequate techniques that could take care of the ecological relationships amongst the Maize-Beans intercrop has not been well elucidated. The objective of the study was to establish economic advantage of maize-beans intercrop when grown under Tithonia and/or synthetic fertilizer.

MATERIALS AND METHODS

The study was conducted at a private farm very close to Kimathi University in Nyeri, Kenya from March to July, 2014 and repeated from August to December, 2014. The area lies at 1815 m above sea level with average minimum and maximum temperatures of 12.2 and 23.2°C, and mean annual rainfall of 928 mm. The soils are well drained, extremely deep with dark reddish brown color, friable clay with an acidic humic top soil (Jaetzold and Schmidt, 2006).

The study was conducted under three cropping patterns consisting of maize alone, maize/beans intercropped, and beans alone. The experimental design was randomized complete block design (RCBD) with four fertilizer treatments and three cropping patterns in three replications. Four fertilizer methods were studied, control without fertilizer (WF); MF 17-17-17 at 150 kg/ha; TDB alone at 8 t ha^{-1} and a combination of MF at 100 kg/ha with TDB at 4 t ha^{-1} ½ (MF+TDB). The experiment was conducted on an area of 33 × 18 m divided into 3 blocks of 18 × 10 m each separated by a 1 m space. The blocks were divided into plots of 3 × 4 m (12 m^2) each with an inter-plot spacing of 0.5 m. Tithonia green leaves were obtained from young tender branches of Tithonia trees, cut and shredded into smaller fragments of less than 5 cm in length with stem girths ranging from 2.8 cm to 4.2 cm enriched with ash, and composted in polythene-aligned pits for three weeks. Initial analysis of soil and chemical composition of Tithonia manure were conducted before planting (Tables 1 and 2).

Three days before planting, TDB was incorporated into the soil at 15 cm depth. Maize (Duma 43) and bean (Mwitemania) seeds from Kenya Seeds Company were sourced from a local agro-supplier in Nyeri. Three maize seeds per hole were sown at a spacing of 0.75 × 0.5 m and 1 × 0.5 m in mono crop and intercrop, respectively. Maize in association plots were intercropped with beans at a spacing of 0.20 m in row. In monoculture, three beans seeds were sown at 0.5 × 0.20 m. Two weeks after sowing (WAS), maize and bean seedlings were thinned to two plants per stand to achieve recommended population of 64 plants per plot (53,333 plants/ha) in sole maize, 240 plants per plot (200,000 plants/ha) in sole bean. In the maize-bean intercropped plots, there were 48 maize plants/plot (40,000 plants/ha) and 160 bean plants/plot (133,333 plants/ha).

Maize was harvested green after attaining physiological maturity and the number of cobs and their weights were recorded from ten randomly selected plants. For the beans, total number of pods and grains per pod per plant were counted from ten randomly selected plants.

LER was used to determine the intercrop advantage as follows:

$$LER = (Yab/Yaa) + (Yba/Ybb) \quad (1)$$

where Yaa and Ybb were yields of sole maize and beans, respectively, while Yab and Yba were crop yields in the maize/bean intercrops for maize and beans, respectively and values of LER greater than 1.0 were considered advantageous (Ofori and Stem, 1987).

Intercropping expected yield of maize and beans was estimated based on the following formula:

$$IEY = MOY \times DIS/DIM \quad (2)$$

Table 1. Initial soil analysis from the experimental site.

Parameter	Units	Value
pH	pH Value	5.2
Organic carbon content	g/kg	39.7
Total nitrogen content	g/kg	3.09
Phosphor stock	mmol P/kg	9.5
K (exch. Potassium)	mmol+/kg	5.6
Mg (exch. Magnesium)	mmol+/kg	35.8
Ca (exch. Calcium)	mmol+/kg	108
Cation exchange capacity	mmol+/kg	178
Clay content	g/kg	720
Sand content	g/kg	70

Table 2. Chemical composition of Tithonia biomass manure.

Parameter	Units	Value
pH	pH value	6.53
Organic carbon content	g/kg	29.57
Total nitrogen content	g/kg	2.5
Phosphor stock	mmol P/kg	0.34
K (exch. Potassium)	mmol+/kg	3.2
Mg (exch. Magnesium)	mmol+/kg	44
Ca (exch. Calcium)	mmol+/kg	60
Cation exchange capacity	mmol+/kg	168

where IEY is the intercropping expected yield, MOY is the mono crop obtained yield for each crop, DIS and DIM are the crop's density in intercropping and mono cropping systems.

To compare the yields of maize and beans, maize yields were converted into bean equivalent yield (BEY) as described by Prasad and Srivastava (1991):

$$\text{BEY (t/ha)} = \text{Yield of maize} \times \text{Unit price of maize/Unit price of bean} \quad (3)$$

The current market price of these two crops was used in calculating BEY in intercrop and in sole. The BEY in intercropping is yield of intercrop beans plus BEY of intercrop maize. The difference between BEY in the intercrop and BEY in the sole represent the agronomic intercropping advantage (AIA) over respective sole crops.

The actual yield loss (AYL) of either maize or beans (AYLa or AYLb) relative to their yield in pure stand was used to calculate the proportion of yield loss or gain of either maize or beans when grown as intercrop according to the formula by Banik et al. (2000) as follows:

$$AYLab = AYLa + AYLb \quad (4)$$

$$AYLa = (Yab/Zab/Ya/Za) - 1 \text{ and } AYLb = (Yba/Zba/Yb/Zb) - 1$$

where Ya and Yb are the yields of maize and beans, respectively, as sole crops and Yab and Yba are the yields of maize and beans in the maize/beans intercrops. Zab and Zba are proportion of maize and beans, respectively. Positive AYL indicates an advantage while negative value indicates disadvantage of the intercrop. This is useful when the main objective is to compare yield on individual plant basis.

To evaluate if the combined yields of maize and beans could be high enough for the farmers to adopt the intercropping system, the economic performance of the two crops grown together was evaluated according to the formula by Ghosh et al. (2006). The higher the MAI value was, the more profitable the cropping system. The MAI was calculated as:

$$MAI = (LER-1) / LER \quad (5)$$

Economic advantage of the intercrop was calculated using the following formula given by Banik et al. (2000):

$$IAma = AYLma \times Pma \text{ and } IAb = AYLb \times Pb \quad (6)$$

where Pma is the commercial value of maize yield (the current price per 110 kg bag of green maize is Ksh 4800), while Pb is the commercial value of beans and the current price per 90 kg bag of beans is Ksh 5400.

Because there were no significant differences among the parameters tested when the data for the two trials were subjected to planned F tests, the data were pooled and analyzed using SAS version 9.1. Significantly different treatment means were separated by Duncan Multiple Range Test at $P \leq 0.05$ levels.

RESULTS

Yields of both sole and mixed crops treated with Tithonia and MF were 24 tons/ha (50% more than the control) and

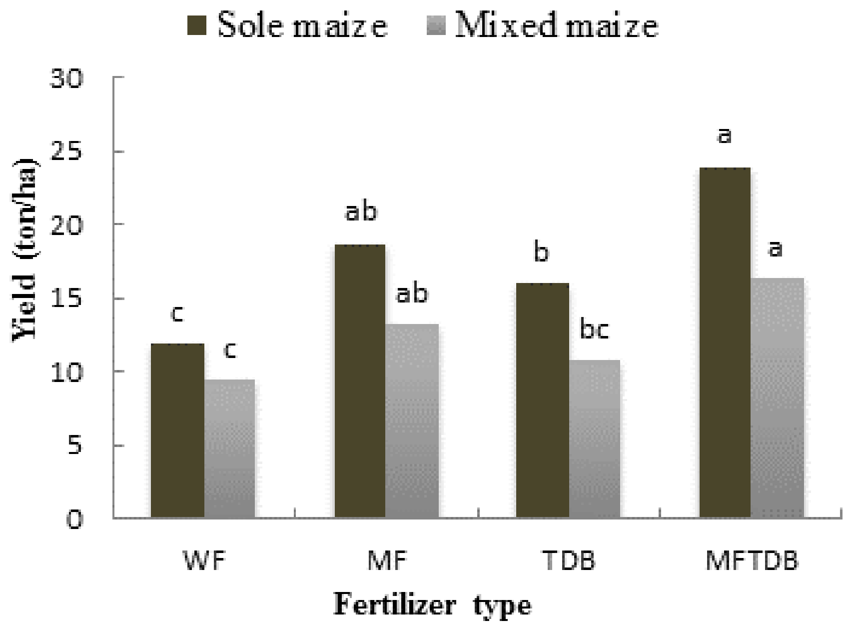

Figure 1. Yield of sole and mixed maize (tons/ha) as affected by Tithonia manure and mineral fertilizer.

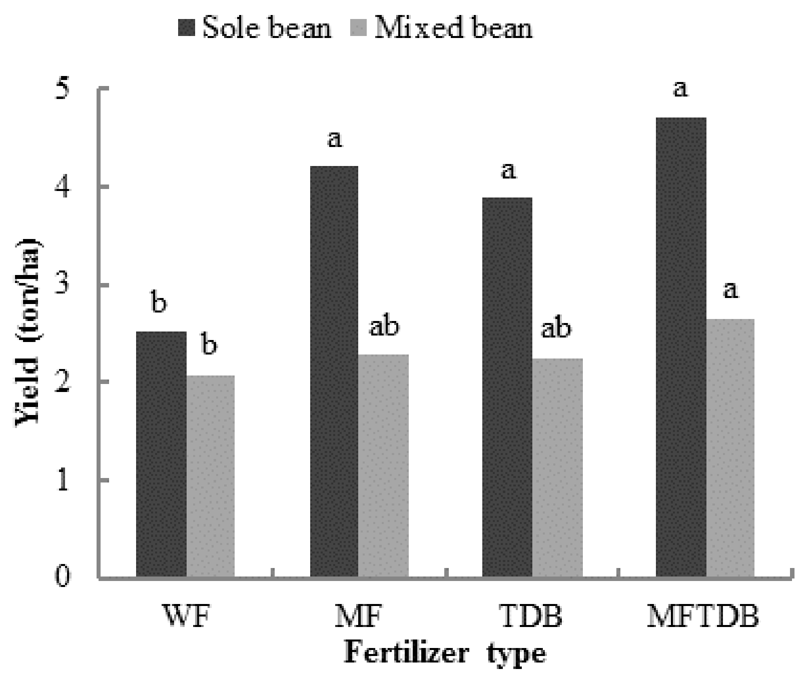

Figure 2. Yield of sole and mixed beans (ton/ha) as affected by Tithonia manure and mineral fertilizer.

16 ton/ha (42% more than the control), respectively (Figure 1). The yields of beans showed a similar trend with application of Tithonia manure and MF. Sole beans performed better than intercropped beans. Integrated nutrient application in mixed crop produced 21.7% higher yields than control, while sole application of MF and TDB produced 8.96 and 7.52% higher yields than the control (Figure 2).

In general, LER for maize and beans was higher than 0.50 in all fertilizer types. LER for maize of 0.83 was the

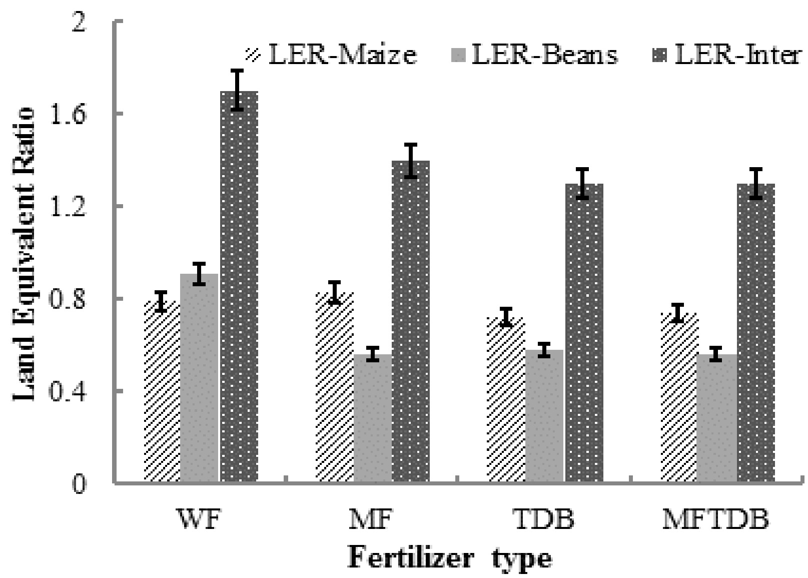

Figure 3. Effect of Tithonia manure and mineral fertilizer application on expected intercropping expected yield.

Table 3. Effect of Tithonia manure and mineral fertilizer on intercropping expected maize and bean yield (tons/ha).

Fertilizer type	IEMY	IOMY	IEBY	IOBY
WF	10.74[a]*	11.34[c]	2.01[b]	2.50[b]
MF	16.84[a]	15.91[ab]	3.37[a]	2.74[ab]
TDB	14.43[a]	12.92[bc]	3.11[a]	2.70[ab]
MF/TDB	21.57[a]	19.55[a]	3.77[a]	3.19[a]

*Means followed by the same letters within a column are not significantly different according to Duncan's Multiple Range Test at P ≤ 0.05. IEMY: Intercropping expected maize yield; IOMY: intercropping obtained maize yield; IEBY: intercropping expected bean yield; IOBY: intercropping obtained bean yield. WF: without fertilizer, MF: mineral fertilizer, TDB: *Tithonia diversifolia* biomass.

highest when MF was used. The control gave the highest LER for beans at 0.91. The lowest LER for maize at 0.72 was obtained when Tithonia manure was used. MF (0.56) gave the lowest LER for beans. However, under intercropping, all the fertilizer types gave a LER of more than 1.0, with the highest and lowest from WF (1.70) and TDB (1.30), respectively (Figure 3)

Intercropping Expected Maize Yield (IEMY) was higher than the intercropping obtained maize yield (IOMY) for all fertilizer types. The shortfall between the expected and obtained maize yield was highest in integrated nutrient application at 1.683 tons. IEMY was not significantly different at P ≤ 0.05 in all the fertilizer types (Table 3). Intercropping Expected Bean Yield (IEBY) was higher than the intercropping obtained bean yield (IOBY) as obtained for all the fertilizer types. The shortfall between the expected and obtained bean yield was highest (0.526 tons) when MF was used (Table 3).

BEY was influenced by application of Tithonia manure and MF in both sole and mixed cropping. The highest BEY in sole cropping was obtained from integrated manure at 20.92 tons/ha. Although fertilizer types did not show significant difference in the BEY; however, a higher yield was recorded for all fertilizer types compared to control. The BEY in the intercrop was higher across the fertilizer types indicating a yield advantage, but the highest BEY was recorded in MFTDB and MF at 24.11 and 19.08 tons/ha, respectively. Although the highest intercrop yield advantage expressed by use of integrated Tithonia manure and MF was 46.45% (Figure 4).

AYL for maize had positive values when MF (0.112) was used and the control had positive values indicating an advantage of the association. The AYL for beans was however negative for all the fertilizer types except for the control (0.3663). The total AYL was all negative for the different fertilizer types indicating an intercrop disadvantage

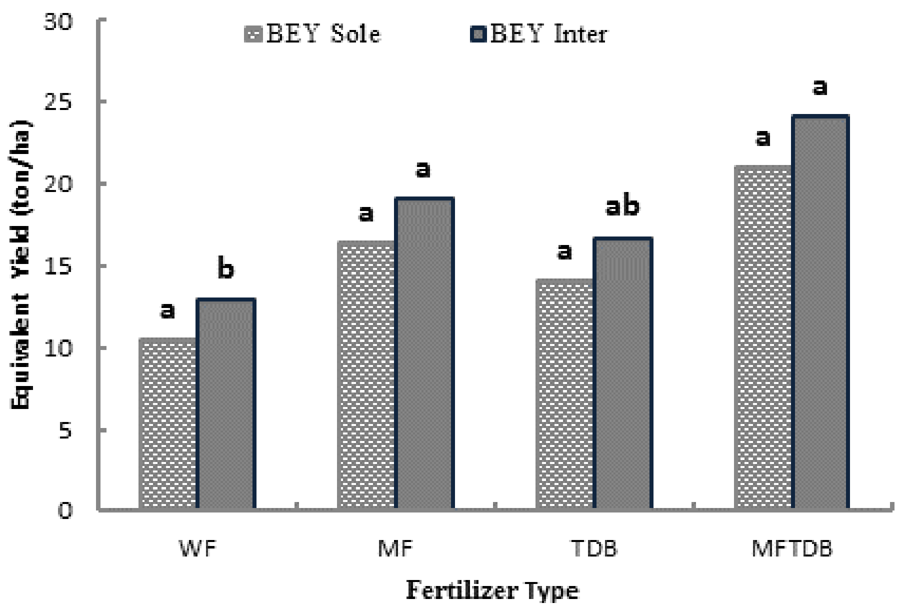

Figure 4. Effect of Tithonia manure and mineral fertilizer on bean equivalent yield.

Table 4. Effect of Tithonia manure and mineral fertilizer on actual yield loss (AYL).

Fertilizer type	AYL_{maize}	AYL_{bean}	AYL
WF	0.0498^a	0.3663^a	0.4162^a
MF	0.112^a	-0.1621^a	-0.0501^a
TDB	-0.036^a	-0.1353^a	-0.1712^a
MF/TDB	-0.0159^a	-0.1534^a	-0.1692^a

*Means followed by the same letters within a column are not significantly different according to Duncan's Multiple Range Test at P ≤ 0.05. AYL: Actual yield loss; WF: without fertilizer; MF: mineral fertilizer; TDB: *Tithonia diversifolia* biomass.

(IA) (Table 4).

IA in maize was 4.89 more profitable where MF was used, but there was an intercrop disadvantage when Tithonia manure was used alone; however, the IA for maize was not significantly different at P ≤ 0.05. There was an intercrop disadvantage for beans in all the fertilizer types. The application of Tithonia manure and MF influenced the MAI. The highest MAI was obtained when integrated manure was used with MAI of 253. Although the means were not significantly different at P ≤ 0.05, but integrated manure application produced 35% more profit than the control (Table 5).

DISCUSSION

The results from this study showed that the highest green maize and bean yields were obtained from the sole cropping plots across the fertilizer types presumably due to the absence of competition from companion crop. However, the combined yields of green maize and beans in the intercropped system were better than the sole yield of either of the two crops. Being a heavy feeder of nutrients, maize productivity is largely dependent on nutrient management. Spatial separation and therefore acquisition of major growth resources at different times in maize and beans could be used to explain the biggest complementary and yield advantage observed (Ofori and Stern, 1987). Another advantage of the intercrop was the complementarity of the maize/bean association as reported by Matusso et al. (2012); that the cereal may be more competitive than the legume for soil mineral N, but the legume fixes N symbiotically making nitrogen available for both crops.

The finding of the present study agrees with many scientists who have worked with cereal-legume intercropping systems (Egbe, 2010; Ghosh et al., 2006; Matusso et al., 2012; Osman et al., 2011) and proved its success compared to mono crops especially for smallholder farmers who aim at minimizing risks against total crop failures and also get different products for the family's food and income. Beans in the intercrop for this

Table 5. Effect of Tithonia manure and mineral fertilizer on the intercropping advantage (IA) and monetary advantage index (MAI).

Fertilizer type	IA maize	IA bean	MAI
WF	2.175[a]*	21.98[a]	202.4[a]
MF	4.889[a]	-9.73[a]	227.1[a]
TDB	-1.569[a]	-8.12[a]	164.0[a]
MF/TDB	-0.692[a]	-9.2[a]	253.4[a]

*Means followed by the same letters within a column are not significantly different according to Duncan's Multiple Range Test at P ≤ 0.05 WF: without fertilizer; MF: mineral fertilizer; TDB: *Tithonia diversifolia* biomass.

present study generally yielded lower than their counterparts in sole which could possibly be due to the shading effects of maize that significantly reduced light interception potential of the associated beans and reduced their photosynthetic assimilation capacity (Ghosh et al., 2006). Reduced photosynthetic assimilation could have resulted in limited food supply for associated *rhizobium* bacteria, thus consequently diminishing their atmospheric fixation capacity.

Application of combined Tithonia manure and mineral fertilizer (MFTDB) produced the highest amount of green maize and beans. These findings were in agreement with those of Aguyoh et al. (2010) who reported a significant and positively correlated increase in total yield of watermelon with increasing application rates of *T. diversifolia* manure, enhancing the yields by between 8.5 and 31% in plants subjected to the highest level of tithonia (5.4 t/ha) compared to the control. Jeptoo et al. (2013) also found out that application of *T. diversifolia* manure resulted in increased total fresh root weight, dry root and shoots biomass and root volume of carrots compared to the control. Rahman et al. (2013) also found out that application of farmyard manure at the rate of 5 tons ha^{-1} contributed about 25 to 30 kg N ha^{-1} to the maize crop.

Integration of nutrients however increased yield and other yield attributes indicating the enhancement of nutrients availability to the plants which could be due to the fact that application of nitrogen in the presence of organic manures helps mineralization process by minimizing C/N ratio. Integrated use of organic manure and chemical fertilizer increased water stable aggregates which could be attributed to the beneficial effects of certain polysaccharides formed during decomposition of organic residues by microbial activity as well as cementing action of bacteria and fungi (Rahman et al., 2013). Growing a legume in the cropping sequence has special significance in the maintenance of soil fertility and crop productivity, because of its unique ability to fix and utilize atmospheric nitrogen.

Intercropping Expected Yield (IEY) of both maize and beans was higher than the Intercropping Obtained Yield (IOY) for all the fertilizer types. The results showed that the individual plant performance was lower in the intercrop and therefore a mutual inhibition and underperformance of both maize and beans in the association was due to crowding, nutrient sharing and shading effects between the maize and bean plants. As it is usually difficult to compare the performance of two different crops in an intercropping system, the yields of maize were converted to the yields of beans; BEY.

The yield advantage in terms of BEY in this study was higher in maize/bean intercropping system than in sole cropping of either crop thereby revealing an agronomic advantage that ranged from 13 to 19%. The yield response due to Tithonia manure and MF was consistent over the study period, where the integrated nutrient application recorded the highest BEY, but the lowest for the control. These findings corroborates the report of Ghosh et al. (2006) who observed a soybean equivalent yield (SEY) of 60% yield advantage from intercropping over sole soybean when sub soiling over conventional tillage was used. Egbe (2010) however found out that SEY figures were not significantly different when different densities of sorghum and soybean were used.

The IA for maize which is an indicator of the economic feasibility of intercropping systems, affirmed that the most advantageous fertilizer type was MF with the highest IA of 4.889. There was intercropping disadvantage for beans for all the fertilizer types except when Tithonia manure was used. IA was depressed when Tithonia manure was used. The present study is in agreement with Yilmaz et al. (2007) who showed intercrop disadvantage at different densities of common bean and cowpea in maize-common bean and maize-cowpea intercrops. Takim (2012) also found out that there was IA for maize and intercrop disadvantage for cowpeas in maize-cowpea intercrop. Beans responded well to Tithonia manure application and had a higher IA than all other fertilizer regimes.

Monetary advantage (MA) of intercropping was used to calculate the absolute value of the genuine yield advantage. Dhima et al. (2007) assuming that the appropriate economic assessment of intercropping should be in terms of increased value per unit area of land. The result showed that the MAI values were positive in all the fertilizer types and therefore a definite yield and economic advantages in maize-bean intercrop over their sole cropping. The highest MAI of 253 was obtained in the integrated nutrient supply with MF/TDB treated plots, which implied that it was the most economical and advantageous fertilizer regime. This could be attributed to the complementarity of the two crops in the mixture. Dhima et al. (2007) obtained the highest MAI values from the common vetch–oat mixture (105.29) at the 65:35 seeding ratio followed by the common vetch-wheat mixture (59.93) at the 55:45 seeding ratio. The author reported that if LER and relative crowding coefficient (K) values were high, then there was an economic benefit expressed with MAI values such as obtained in this study.

Conclusions

The study proves that the use of Tithonia manure and/or MF (17:17:17) affect growth and yield of maize and beans whether grown alone or in a mixture. The intercropping of maize and beans, regardless of the fertilizer regimes has agro-biological advantages over individual crops. The LER were higher than one in all intercropping plots indicating an optimum exploitation of the environmental resources. The yield advantage in terms of BEY was higher with an agronomic advantage of 13 to 19%. Generally, maize dominated beans except when Tithonia manure was used. The IA and MAI indicated a definite yield and economic advantages in maize-bean intercrop over their sole cropping.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Storage of *Alibertia edulis* seeds: Influence of water content and storage conditions

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Knowledge of the desiccation sensitivity of *Alibertia edulis* seeds is essential to provide adequate conditions for maintaining viability during storage. Thus, the objective of this study was to evaluate the effect of variations in the water content, environmental conditions, and storage periods on the conservation of *A. edulis* seeds. After processing, the seeds were dried under ambient conditions to water contents of 20, 15, 10 and 5±2%, and then subjected to storage under laboratory (25°C), cold chamber (16°C), refrigerator (8°C), and freezer (-18°C) conditions for zero, 30, 60, 90, 120, 150 and 180 days. To assess the physiological potential of the seeds, protrusion of the primary root, percentage of normal seedlings, germination speed index, total seedling length and total dry mass of seedlings were performed. A completely randomized factorial split-plot design (4 water contents × 4 temperature × 7 storage periods) was used for the experiment. *A. edulis* seeds tolerated water content reduction to 5% and storage under room temperature and cold chamber storage conditions for 150 days. Seeds with water content between 10 and 5% did not tolerate more than 60 days of freezing conditions, confirming their physiological behavior as intermediate.

Key words: Brazilian savanna, conservation, drying, Rubiaceae.

INTRODUCTION

The Brazilian Savanna holds 5% of the planet's biodiversity and is considered the richest savanna in the world, but one of the most threatened biomes (MMA, 2010). Unfortunately, many of the natural resources present in this biome, such as medicinal plants and fruit species, some endemic (Klink and Machado, 2005), are disappearing because of uncontrolled deforestation.

Many of these native species could be used to produce seedlings, among other objectives, to recover or enhance degraded areas and maintain germplasm banks to guarantee the conservation of biodiversity (Martins et al., 2009). Seed storage is of fundamental importance in the preservation of the physical, physiological, and health qualities of seeds, ensuring the conservation of the genetic diversity of many species for scientific research and agriculture (Medeiros and Eira, 2006). However, the

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success of storing seeds depends on understanding their behavior during the process, which enables the use of appropriate conditions for maintaining their viability (Hong and Ellis, 1996).

Regarding their behavior in storage, seeds are usually classified into two distinct groups: orthodox and recalcitrant. Orthodox seeds remain viable after drying to a moisture content of down to 5% and remain viable for long periods at low temperatures. Recalcitrant seeds are sensitive to desiccation and do not survive under low levels of moisture, which prevents their long-term storage (Roberts, 1973). Besides these groups, there is a third group of seeds with intermediate storage behavior (Ellis et al., 1990). Intermediate seeds tolerate dehydration of 7.0 to 10% moisture and do not tolerate low temperatures for extended periods of time (Hong and Ellis, 1996).

Tolerance to desiccation is one of the most important properties of seeds, and is defined as the ability to survive without irreversible damage after the complete or almost complete removal of intracellular water (Leprince and Buitink, 2010). Several mechanisms have been associated with desiccation tolerance in seeds, such as: reduction in the degree of vacuolation; the accumulation of insoluble reserves; reaction of the cytoskeleton; nuclear DNA conformation; intracellular differentiation; metabolic shutdown; presence and efficiency of antioxidant systems; and accumulation of protective molecules (Berjak and Pammenter, 2001). However, further studies are needed to determine the behavior of seeds of native tropical species in terms of drying, duration of drying, and necessary storage conditions for maintaining the physiological potential of seeds, in order to conserve the germplasm of species occurring naturally in the Savanna.

Alibertia edulis (Rich.) A. Rich. ex DC. (Rubiaceae), popularly known as Apuruí, Marmelada-Nativa, Marmelão, and Marmelo do Cerrado, has nutritional and medicinal importance. The fruits are globular shape with size of about 2 to 4 cm long and 2 to 4 cm diameter black color when ripe and greatly appreciated by the public, can be consumed fresh or as jelly, sweets and soft drinks (Silva et al., 2001; Chiquieri et al., 2004). The roasted seed is used to replace coffee, and the fruit can also be fed to livestock (Felfili et al., 2000).

There is some information in the literature regarding the occurrence of desiccation sensitivity in the seeds of other species of Rubiaceae, as reported for *Coffea canephora* Pierre ex A. Froehner; its seeds showed tolerance to desiccation down to 15% moisture and could be stored for four months at 10°C (Rosa et al., 2005); and for *Coffea arabica* and *Coffea congensis* A. Froehner, where desiccation up to levels less than 9% can damage the seeds, thus demonstrating the sensitivity of *Coffea* species to desiccation (Eira et al., 2006). *Genipa americana* L. seeds dried to 10 and 5% moisture had reduced viability after 30 days of storage, but there was no total loss of germination after this period (Salomão, 2004; Magistrali et al., 2013).

Thus, the lack of knowledge regarding the longevity of *A. edulis* seeds hinders the exploitation of the properties of fruits and optimization of the cultivation of this species for commercial purposes, in reforestation, and maintenance in germplasm bank programs. The hypothesis of this study is that seeds of *A. edulis* can be stored for a period exceeding 30 days at low temperatures provided their water content is maintained around 5%.

To test this hypothesis and to determine the physiological behavior of the seeds of this species, this study evaluated the effect of different water contents, environmental conditions, and storage periods on the physiological potential of *A. edulis* seeds.

MATERIALS AND METHODS

Vegetal material

A. edulis fruits were collected at the end of July, 2013 from 15 arrays located in an region of the Savanna (*sensu stricto*) in Dourados - MS. After collection, the fruits were brought to the Laboratory of Nutrition and Metabolism of Plants, Universidade Federal da Grande Dourados (UFGD) in Dourados - MS, where they were washed in running water and any damaged fruits were discarded. Subsequently, the fruits were processed manually and on sieves to separate the seeds. The seeds were then washed with tap water and placed on Germitest® paper for 40 min at room temperature (25°C±2°C, 32% relative humidity) to remove excess moisture.

After water was removed from the seed surfaces, the seeds were dried under laboratory (25°C) conditions on plastic trays and weighed until they reached the pre-established water content (20, 15, 10 and 5%, respectively), as calculated according to the formula of Sacandé et al. (2004). To obtain 20, 15, 10, and 5% moisture content water, 7, 25, 28, and 30 h of drying, respectively, were required at room temperature.

When the water content levels of the seeds were found to be near those desired, samples were taken, homogenized, and divided into fractions packaged aluminum foil bags. These samples were subjected to the following storage conditions: 25±2°C, 35% relative humidity (laboratory); 16±2°C, 40% relative humidity (cold chamber); 8±2°C, 35% relative humidity (refrigeration); and -18±2°C, 42% relative humidity (freezing). After 0 (newly processed), 30, 60, 90, 120, 150, and 180 days of storage, the seeds were pre-humidified to 100% relative humidity and 25°C under continuous white light for 24 h, thereby preventing damage from soaking.

Evaluation of seed physiological quality

The following physiological characteristics were then determined. The water content was determined at 105°C±3°C for 24 h using the oven-drying (Brasil, 2009) with three replicates of 5 g of seeds each, expressed as percentage on a fresh weight basis.

Protrusion of the primary root was measured on Germitest® paper rolls with four replications of 25 seeds each, which were kept in B.O.D. (Biochemical Oxygen Demand) at 25°C under continuous white light. Assessments were conducted daily, and the root was considered protruded when it reached a length of 5 mm. The results were expressed in percentages (%).

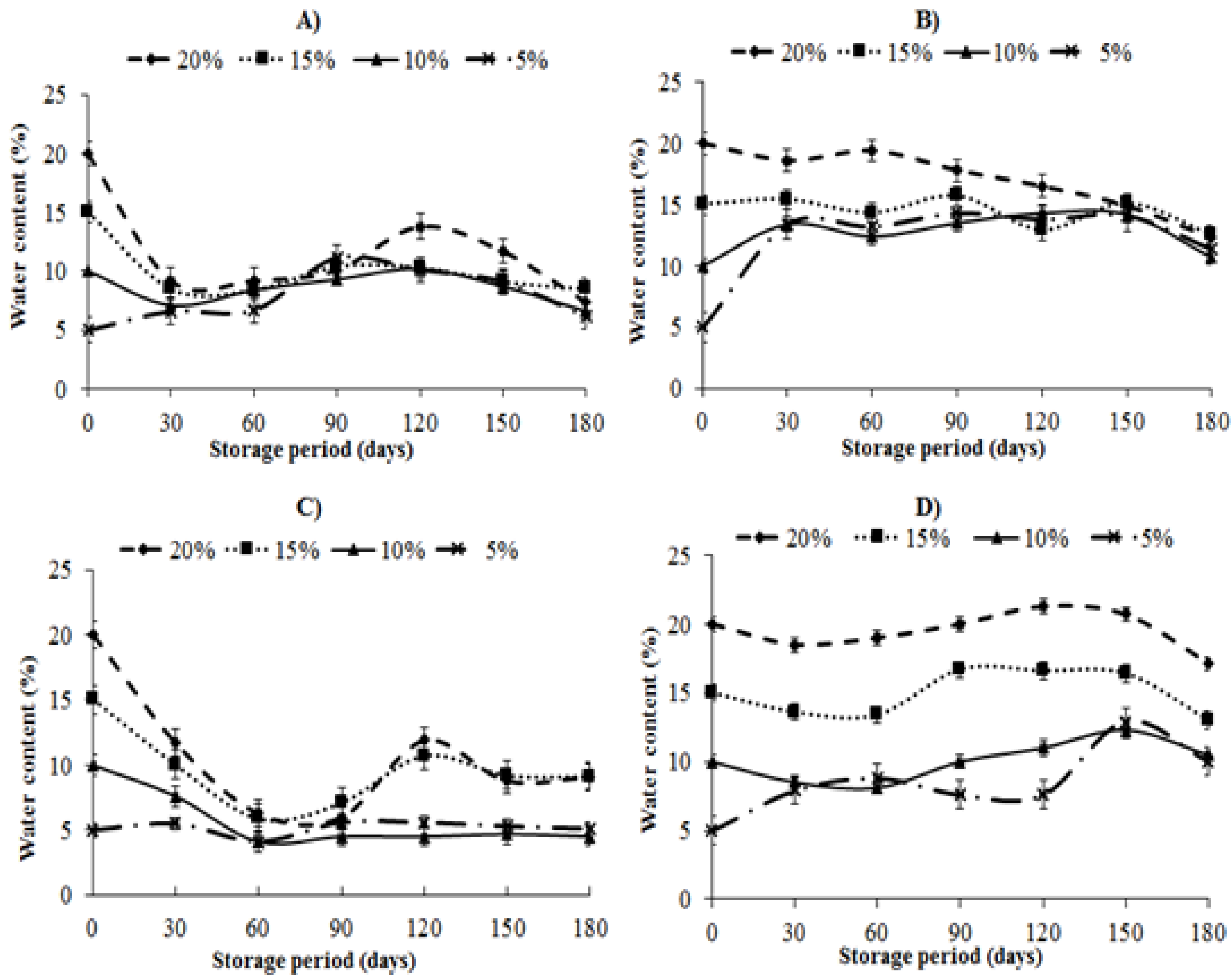

Figure 1. Water content (%) of *A. edulis* seeds packed with different water contents (%), environmental temperature (A - Laboratory, B - cold chamber, C- refrigerator and D - freezer) and stored for different periods.

The percentage of normal seedlings was determined in Germitest® paper rolls with four replications of 25 seeds each, which were put in BOD at 25°C under continuous white light. Evaluations were performed forty-five days after sowing by computing the percentages of normal seedlings, using the issuance of shoots and root system development. The results were expressed in percentages (%).

Germination speed index (GSI) was calculated using the numbers of seedlings that germinated each day divided by the numbers of days between sowing and germination, according to the formula proposed by Maguire (1962). Seedling length was obtained by measuring the lengths of the primary root, shoot and total plant using a millimeter ruler. The results were expressed in centimeters (cm $seedlings^{-1}$).

The total dry mass was obtained from seedlings that had been dried in an oven at 60°C for 48 h using an analytical balance (0.0001 g), with the results expressed in grams (g $seedlings^{-1}$).

Experimental design/ statistical analysis

A completely randomized factorial split-plot design (4 temperature/environment conditions × 4 water contents × 7 storage periods) was used for the experiment. For data showing significance in the analysis of variance, the temperature data were compared by a Tukey's test and the data on water content and storage period were adjusted by regression equations at 5% probability using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Seeds stored under laboratory conditions (25°C), a cold chamber (16°C), a refrigerator (8°C) and a freezer (-18°C) showed variations in the water content during storage (Figure 1). The variations observed in the water content of seeds stored under different conditions evaluated over 180 days demonstrated it can be a semi-permeable packing, there was an exchange of water vapor between the seed and the external environment, thus changing the level of hydration of the seeds during storage. However, this type of semi-permeable packaging

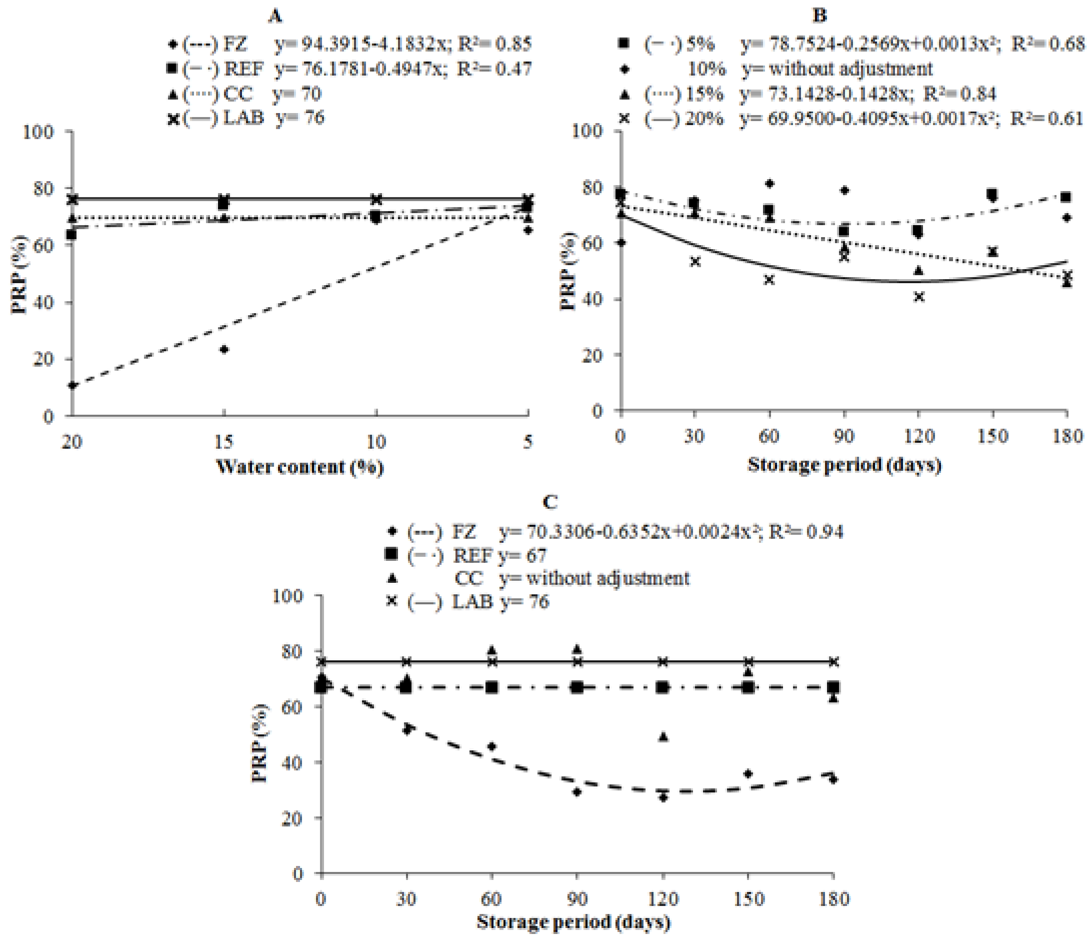

Figure 2. Primary root protrusion (PRP) (%) of *A. edulis* seeds according interactions of water content x storage conditions (A), storage periods x water content (B) and storage periods x storage conditions (C) LAB (Laboratory), CC (cold chamber), refrigerator (REF) and freezer (FZ).

is often used for the storage of seeds in banks. It is noteworthy that the quality of packaging varies, but it also highlights the tremendous opportunities to customize packaging properties by combining thermal plastics and water vapor barriers, and by manipulating layer thicknesses (Gómez-Campos, 2006; Walters, 2007).

In laboratory, cold chamber and refrigerator conditions, significant reductions were observed in the water content after 30 days of storage, mainly for the 20 and 15% water content. The seeds stored under freezer conditions showed little variation in the levels of 20, 15, 10, and 5% water, presenting at the end of the 180 days storage mean results of 17, 13, 11, and 9%, respectively.

For the protrusion of the primary, there were significant interactions for water contents × storage conditions, storage period × storage conditions, and storage periods × water contents (Figure 2). With the water content used, the storage conditions of higher temperatures such as laboratory temperature (25°C) and cold chamber (16°C) gave average values of 76 and 70% germination data respectively. Under the refrigerator conditions (8°C), the primary root protrusion increased as the water content was reduced, and this behavior was most significant in freezing conditions (-18°C) (Figure 2A).

In the interaction between storage period and water content, the quadratic equation the water content of 5% showed a minimum value for primary root protrusion of 67% at 92 days of storage, although no differences were observed during the storage time for the seeds with a water content of 10% (Figure 2B). The different storage conditions did not affect primary root protrusion over the 180 days of storage, except under freezing conditions showing minimum values of 30% at 128 days (Figure 2C).

The triple interaction (water content × storage conditions × storage period) was significant for the percentage of normal seedlings and germination speed index (GSI), this being represented by the split depending on the storage conditions. Under laboratory storage conditions (25°C), the maximum percentage of normal

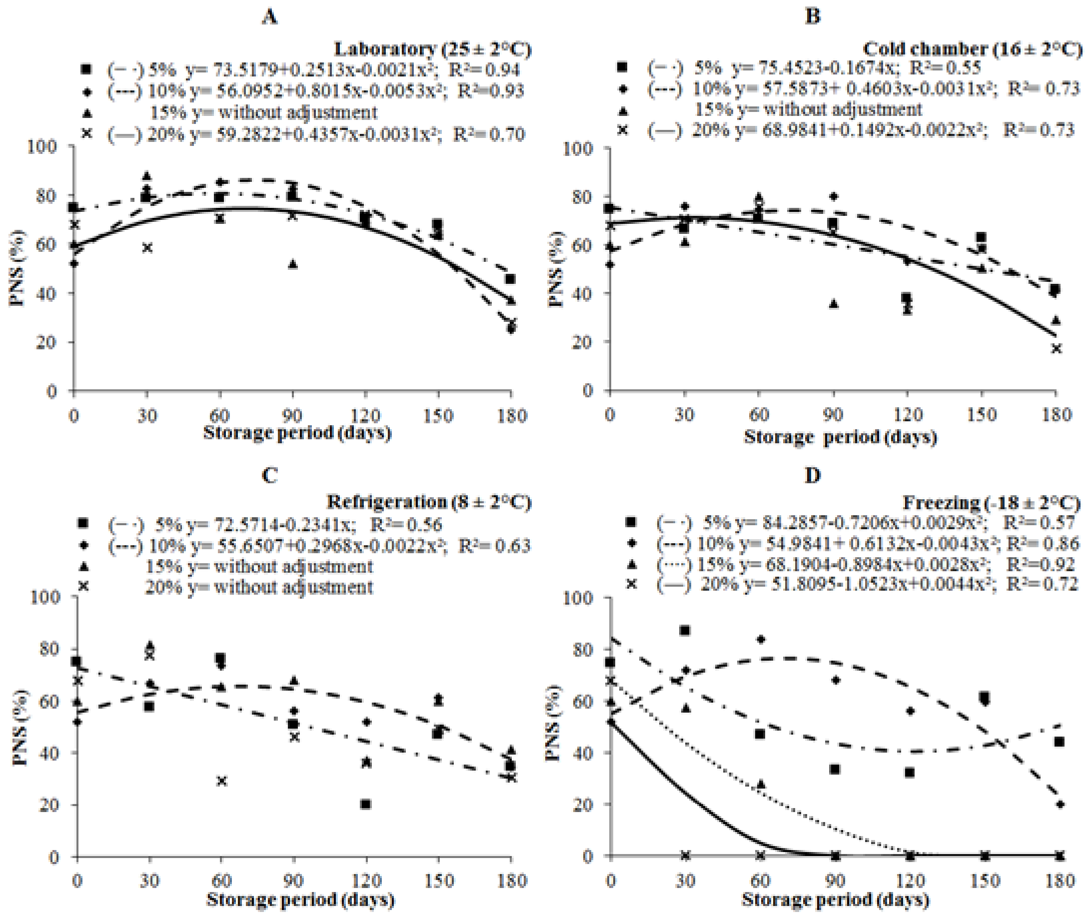

Figure 3. Percentage of normal seedlings (PNS) (%) of *A. edulis* according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

seedlings was observed for seeds with a water content of 10% (86%) after 75 days of storage, followed by seeds stored with a water content of 5% (82%) after 54 days of storage (Figure 3A).

For seeds stored under cold chamber storage conditions (16°C), the maximum percentage of normal seedlings was observed for seeds with a water content of 10% (74%) after 73 days of storage (Figure 3B). Seeds stored with initial water content of 5 and 20% showed a reduction in the percentage of normal seedlings over the 180 days of storage. Seeds stored under refrigerator conditions (8°C) showed a maximum percentage of normal seedlings after 67 days of storage (66%) at 10% (Figure 3C). For freezing conditions (-18°C), the highest percentage of normal seedlings (76%) was observed in seeds stored with a water content of 10% after 70 days of storage (Figure 3D). However, seeds with 5% water content had higher than 50% normal seedlings for up to 60 days of storage and the seeds with water content of 15 and 20% had values below 50% at 30 days of storage.

The classification criterion which distinguishes intermediate and orthodox seeds is seed storage after drying to 5% moisture content at -20°C for a period of 90 days in order to maintain viability (Hong and Ellis 1996). However, it has been reported that an important feature related to intermediate seeds of tropical origin is the fact that their longevity is reduced when stored at temperatures below 10°C (Ellis et al., 1991; Hong and Ellis, 1992). Thus, it was demonstrated that *A. edulis* seeds tolerated a reduction in water content between 5 and 10%, and remain viable during 150 days of storage; however, they lost viability when exposed to freezing temperatures (-18°C) during storage, indicating that they can be classified as intermediate as far as drying and storage is concerned.

The sensitivity to freezing was observed in seeds of other species of Rubiaceae; Coffee seeds are now considered to have storage behavior defined as intermediate (Ellis et al., 1990, 1991; Hong and Ellis, 1996). Seeds of *Genipa americana* L. (Rubiaceae) dried to water contents between 10 and 5% and stored at -20°C showed high percentages of germination and normal seedlings for up to 30 days of storage, and after this period showed complete loss of viability, indicating

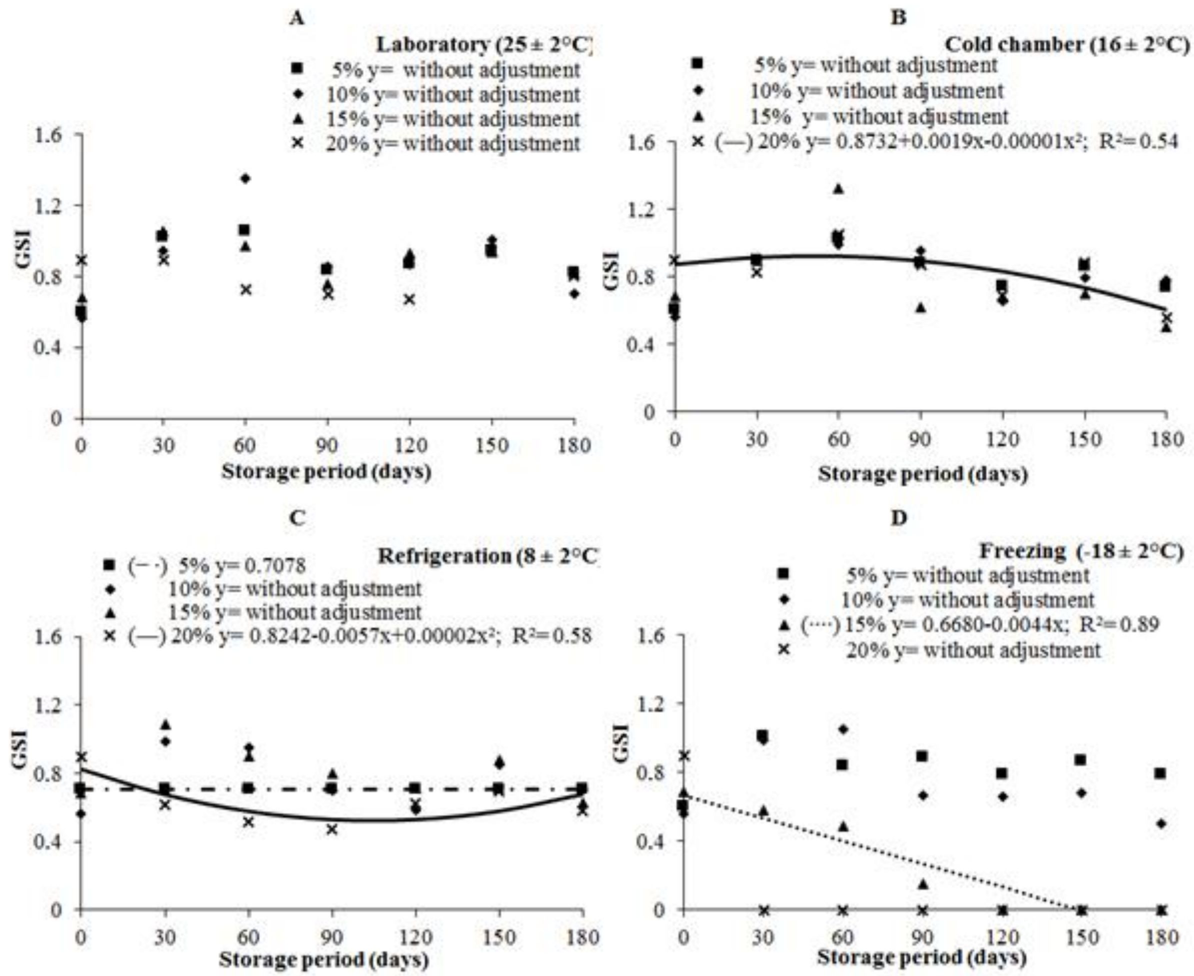

Figure 4. Germination speed index (GSI) of *A. edulis* seeds according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

an intermediate behavior in relation to drying and storage (Magistrali et al., 2013). *Alibertia patinoi* (Cuatrec.) Delprete & C.H. Perss. (Rubiaceae) seeds were classified as recalcitrant, and have high water content (44%) and exhibit continuous metabolism even after dispersion (Escobar and Torres, 2013). Drying of seeds can cause extensive ultrastructural damage to cells, including protein denaturation, crystallization of solutes, and damage to the membranes (Black et al., 2002). Thus, the ability to maintain cellular integrity and repair damage caused by drying is of fundamental importance for the seeds to tolerate desiccation (Pammenter and Berjak, 1999).

The sensitivity of the *A. edulis* seeds at low temperatures (-18°C, freezer) was more pronounced in seeds stored with higher water content (15 and 20%), with the formation of normal seedlings for only up to 30 days of storage. Possibly, the intracellular water formed ice crystals during storage, causing damage to the cellular compartment; thus, the seeds lost viability when rehydrated. Damage caused by freezing may be due to membrane lipid peroxidation promoted by both dehydration and freezing (Wen et al., 2012); however, intracellular ice formation per se is not lethal, at least for the size and density of crystals (Wesley-Smith et al., 2014).

Regarding the germination speed, seeds stored under laboratory conditions (25°C) did not show significantly adjusted equations (Figure 4A). Under cold room conditions (16°C), the maximum germination speed was recorded after 51 days of storage in seeds stored with an initial moisture content of 20% (0.92) (Figure 4B). Seeds stored in a refrigerator (8°C) with an initial moisture content of 5% showed a medium germination speed of 0.70 over the 180 days of storage (Figure 4C), although for the water content of 20%, a minimum GSI value (0.52) was observed for 106 days of storage (Figure 4C). Freezer conditions (-18°C) negatively affected the germination rate of seeds stored with an initial moisture content of 15%, being terminated at 150 days (Figure 4D).

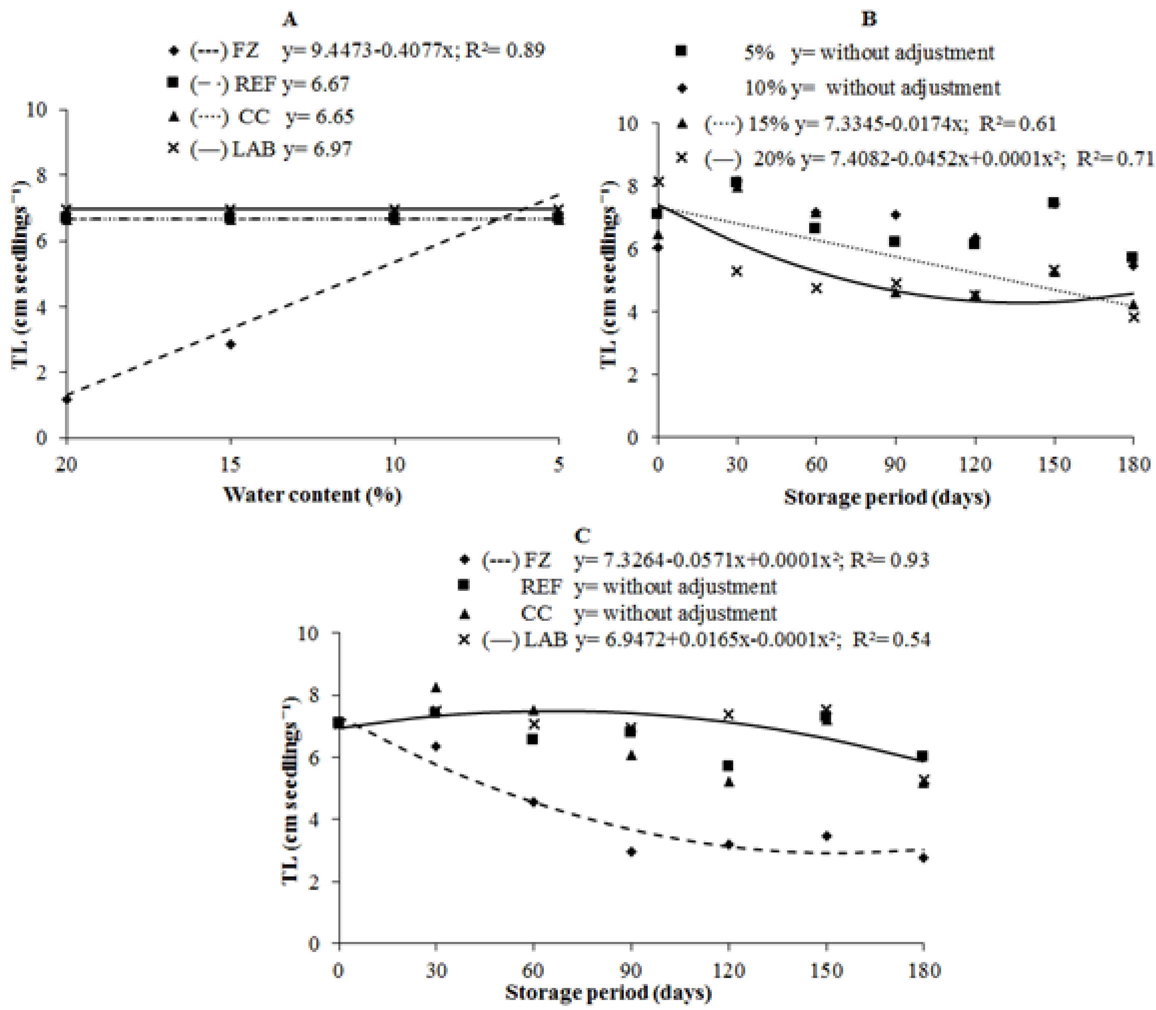

Figure 5. Total length (TL) (cm seedlings⁻¹) of *A. edulis* seedling according interactions of water content x storage conditions (A), storage periods x storage conditions (B) and storage periods x water content (C). LAB (Laboratory), CC (cold chamber), refrigerator (REF) and freezer (FZ).

The interaction of water content × storage conditions, storage period × storage conditions, and storage periods × water content were significant for the total seedling length (Figure 5). Seeds stored under freezing conditions (-18°C) with an initial moisture content of 5% had higher seedling growth throughout the storage period (7.40 cm seedlings⁻¹) (Figure 5A).

For interaction of water with storage periods, there were no differences in total seedling growth over the 180 days of storage for seeds stored with a water content of 5 and 10% (Figure 5B); however, for seeds stored with a moisture content of 20%, the minimum value observed for total seedling growth (4.28 cm seedlings⁻¹) was at 138 days of storage (Figure 5B).

Seeds stored under laboratory conditions (25°C) showed maximum total growth (7.49 cm seedlings⁻¹) after 66 days of storage (Figure 5C). Under cold chamber (16°C) and refrigerator conditions (8°C), there were no differences in total seedling growth over the 180 days of storage; however, under freezing conditions (-18°C), a significant reduction in seedling growth was verified, while a minimum length (2.91 cm seedlings⁻¹) was observed at 154 days of storage (Figure 5).

The triple interaction (water content × storage conditions × storage period) was significant for the accumulation of total dry matter (TDM), this being represented by the split depending on the storage condition (Figure 6). With respect to the accumulation of total dry mass, seeds stored under laboratory (25°C) (Figure 6A) and cold chamber (16°C) (Figure 6B) conditions with an initial moisture content of 5% had a significant accumulation of total dry mass of 0.0054 g seedlings⁻¹ over the 180 days, evidencing the translocation of reservations for the formation of seedlings. On the other hand, seeds stored with a water content of 10 and 20% under laboratory conditions

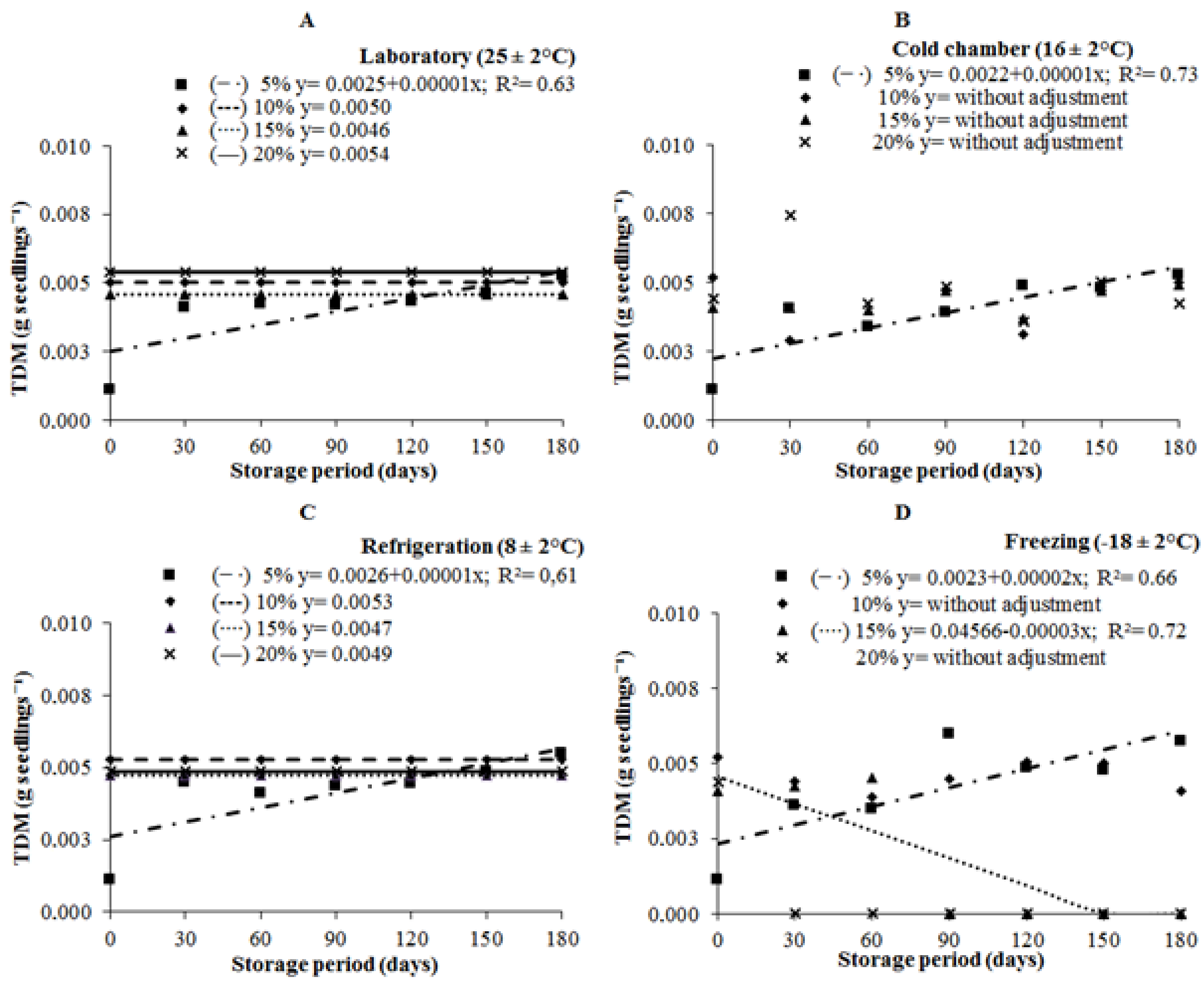

Figure 6. Total dry mass (TDM) (g) of *Alibertia edulis* seedlings according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

(25°C) yielded an average accumulation of dry matter of 0.0050 g seedlings^{-1} over the 180 days of storage (Figure 6A).

Seeds stored under refrigerator conditions (8°C) with an initial moisture content of 5% had a significant increase in the average accumulation of total dry mass (0.0050 g seedlings^{-1}) over 180 days of storage; however, the seeds with 10% water content had an average accumulation of 0.0053 g seedlings^{-1} throughout the storage period (Figure 6C). Freezing conditions (-18°C) negatively influenced the accumulation of seed mass stored with 15% water content, totally ceasing at 150 days (Figure 6D). On the other hand, seeds stored with 5% water content had an average accumulation of total dry mass of 0.0050 g seedlings^{-1} at 180 days of storage.

The negative effect of seed storage with high water content (15% and 20%) under low temperature conditions (-18°C freezer) was also observed in other parameters such as germination speed index, which stopped at 150 days (content of 15% water) and 30 days (content 20% water) of storage (Figure 4D), causing reduction in seedling growth, and thus interfering with the accumulation of total dry mass (Figure 5 and 6D). These results, together with the percentages of radicle protrusion and normal seedlings, suggest that the nature of the damage due to freezing is different from the mechanisms of damage caused by the drying of the seeds in water contents between 10 and 5%. Although the seeds of *A. edulis* demonstrated some tolerance to desiccation, storage at temperatures below 10°C adversely affected the maintenance of the longevity of these seeds, confirming the intermediate physiological behavior of the seeds.

Therefore, it is evident that seeds with intermediate behavior cannot be stored in accordance with the protocols usually recommended for the storage of seeds, because although they apparently survive conditions of low water content, they do not survive the additional stress of exposure to -18°C (Black et al., 2002). The major impediment to storing seeds with intermediate physiologies (that is, Coffee) is an understanding of the limit to which seeds can be dried and the interaction of temperature and water content on seed survival. For non-orthodox seed species, cryopreservation is the only technique available for long-term germplasm conservation. In the case of intermediate seed-propagated species, seeds are partially desiccation tolerant and, therefore, the option which has to be always tested first is whole-seed cryopreservation (Eira et al., 2006).

Thus, it is worth mentioning the importance of prior knowledge of the physiological behavior of the seeds to determine the proper technique for safe storage. This aspect becomes more relevant for native species from threatened biomes that require specific criteria for their long-term storage, such as the species investigated in the present work. Accordingly, the reduction of water content associated with favorable storage conditions is crucial for maintaining the physiological quality of seeds of *A. edulis*. For seeds that are sensitive to drying and storing, maintenance of viability is still one of the biggest challenges faced by researchers for conservation in gene banks.

One of the considerations for the physiological behavior of *A. edulis* seeds is the ecological relationship with the habitat (Daws et al., 2004; Kochanek et al., 2010). From an ecological perspective it is worth noting that seeds classified as intermediate are essentially desiccation tolerant (Tweddle et al., 2003). In terms of habitat, most sensitive seeds (89%) occupy wet forest, riverine, flooded, or coastal environments. Most species (79%) are native to the tropics and many of the desiccation-intolerant species produce seeds that mature during tropical monsoons and rainy seasons, and are unlikely to experience dry conditions (Farnsworth, 2000).

The effects of water content and temperature are interdependent and therefore, current results of *A. edulis* show that critical water content always increases with decreasing temperature. So, there is a narrow window of seed moisture status at which the storage of *A. edulis* seeds is feasible. Just as it was reported the occurrence of intermediate behavior in seeds of other species of the family Rubiaceae, further research on the different levels of desiccation sensitivity among others *Alibertia* species should result in a better understanding of seed longevity and appropriate commercial seed and germplasm storage protocols.

Conclusions

A. edulis seeds tolerate a reduction in their water content to 5% and room temperature (25°C), and cold chamber (16°C) storage conditions for 150 days. Seeds with water content of between 10 and 5% did not tolerate more than 60 days under freezing (-18°C) storage conditions, confirming their physiological behavior as intermediate. The storage of seeds with high water content (15 and 20%) in conditions of low temperature (-18°C freezing) reduced seedling growth and accumulation of total dry mass.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of irrigated and rain fed conditions on infestation levels of thrips (Thysanoptera: Thripidae) infesting *Dolichos lablab* (L.) in Eastern Kenya

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***Dolichos lablab* (L.) is a neglected crop species in Kenya, grown only for subsistence where they are highly adapted to a small range of ecological niches. It is grown under irrigation and rain fed conditions, where production is limited by insect pests such as thrips (Genera: *Megalurothrips* and *Frankliniella*). However, the effects of irrigation and rain fed conditions on infestation levels of these thrips in Kenya are unknown. This study aimed at investigating the levels of infestation of thrips on *D. lablab* (L.) grown under rain fed and irrigated conditions in Meru Central and Yatta sub-counties, Kenya. Sampling was conducted biweekly from June, 2009 to February, 2010. In each sub-county, eight farms (four each irrigation and rain-fed) were randomly selected. The infestation levels were monitored using 5 quadrats (30 cm × 30 cm), placed randomly at each farm in every sampling session. The thrips population within each quadrat was estimated from 3 randomly selected shoots of *D. lablab.* Thrips samples were placed in 30 ml vials and transported in a cool box to the International Centre for Insect Physiology and Ecology (ICIPE) labs for sorting and identification. The abundance of thrips varied significantly between the irrigated and rain fed farms at both sub-counties. In Meru sub-county, thrips numbers averaged 352.3±36.1 for irrigated farms as compared to 199.1±26.3 for rain fed. In Yatta sub-county, the mean abundance for rain fed farms was 265.5±42.0 as compared to 235.4±37.2 for irrigated farms. *D. lablab* for irrigated farms were significantly more infested (Anova: p=0.05) than in rain fed farms.**

Key words: Infestation levels, sampling, irrigated, rain fed, quadrats.

INTRODUCTION

Dolichos lablab (L.), also known as *Lablab purpereus* (L.) is a neglected and under cultivated legume crop in Kenya and other parts of the world (Williams and Haq, 2002), as it is grown only in local production systems, where they

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are adapted to a small range of ecological niches and are highly underdeveloped due to lack of formal research input (Padulosi et al., 2006). In Kenya, the main *D. lablab* producing areas are Eastern, Central and Coast regions, where it's grown either as a pure stand or as an intercrop with maize (Ministry of Agriculture, Kenya Government, 2005).

D. lablab is a drought resistant and highly nutritious crop, but production is severely limited by pest infestations, especially thrips. The few farmers growing the crop have had decreasing levels of attainable yields due to pest infestations, with production ranging from 800 to 900 kg ha^{-1}, which is far less than the estimated yield potential of 2700 to 3000 kg ha^{-1} (Kamau et al., 2010; Kinyua et al., 2008). As a result of the low production, *D. lablab* is often being replaced with other legume crops such as common and French beans, which may not withstand the harsh environmental conditions in such areas.

In Kenya, *D. lablab* is grown under both irrigation and rain fed conditions. In the eastern region, the vegetable type is grown under irrigation while the grain type relies on rains. The vegetable type is locally known as *Varole* and is a perennial type grown mainly for its young fresh pods. It is trellised and climb on the stakes up to three meters high before it starts growing horizontally. It remains ever green throughout the production period as long as there is water for irrigation. The grain type on the other hand is annual and mainly grown for its fresh or dry grains. It is drought resistant and remains ever green during the dry season long after other crops have dried (Maundu et al., 1999). In both sub-counties, rains have been reported to be erratic and unreliable with annual average ranging between 500 to 800 mm (Wambugu et al., 2010)

The proliferation of insect pests such as thrips is more favorable in warm climates (Anonymous, 2006). In such climates, irrigation can stimulate the insect pest population levels (Kannan and Mohammed, 2001). In India, production of *D. lablab* is limited by a wide range of insect pests, but thrips were found to be the major insect pests in both irrigated and rain fed conditions (Thejaswi et al., 2007; Rekha and Mallapur, 2009). For *D. lablab*, irrigation can extend the growing seasons which enables insect pests to complete a greater number of reproductive cycles (Kannan and Mohammed, 2001). This can lead to increased pest infestations, with farmers needing to apply higher levels of chemical pesticides with an aim of controlling *D. lablab* pests, particularly thrips.

In Kenya, information on infestation levels of thrip species associated with *D. lablab* in irrigated and rain fed conditions is lacking. However, thrips of the genus *Frankliniella* and *Megalurothrips* have been reported to infest *D. lablab* in the eastern region of Kenya (Kamau et al., 2010). These genera were found in young leaves, flower buds, flowers and young pods. In India, rain fed and irrigated *D. lablab,* severe infestation was characterized by flower and pod malformation, distortion and scarification, while flower buds did not open but aborted prematurely (Thejaswi et al., 2007).

This study aimed at investigating the effects of irrigated and rain fed conditions on the thrips species and their infestation levels in D*. lablab*.

MATERIALS AND METHODS

Thrips infestation levels on D. *lablab* were evaluated biweekly throughout the two seasons in Yatta (37°53' E and 1°56'S) and Meru Central (37°30'E and 2°50'S) Sub-counties, in the eastern region of Kenya (Figure 1). In Yatta, rains were reported to be erratic and unreliable with annual average ranging between 500 to 800 mm (Wambugu et al., 2010) while in Meru, the annual average received was as low as 500 mm. The average lowest and highest temperatures in Meru were recorded as 16 and 23°C respectively (Oginosaka et al., 2006).

Average minimum and maximum temperatures for Yatta were 13.8 and 30.7°C sequentially (Kang'au, 2011). Studies were carried out under farmers' field conditions during the dry (June, 2009 to October, 2009) and wet seasons (November, 2009 to February, 2010). In both sub-counties, *D. lablab* was grown under irrigation and rain fed conditions. Four-irrigated (vegetable type) and four rain-fed (grain type) farms were randomly selected in each sub-county, totaling 16 farms (farms 1 to 8 in Meru and 9 to 16 in Yatta) in the 2 sub-counties. At each farm, thrips infestation levels were assessed from a set of 4 randomly selected *D. lablab* plants; which were selected from five randomly selected points on the study farms. The sampling of *D. lablab* was done at the seedling and vegetative stages, before the twigs intertwined to form a single canopy during every sampling session. Infestation levels were further assessed at the vegetative and podding stages, when the crop twigs intertwined and formed a dense canopy using a 30 cm x 30 cm quadrat (0.09 m^2).

From each set of four *D. lablab* plants or the plant parts within the quadrat, thrips populations were estimated by picking three randomly selected shoots, and their leaves, flowers and flower buds placed in 30 ml vials. The thrips samples were taken to ICIPE laboratories in Nairobi for sorting and identification. In the laboratory, the leaves, flowers and flower buds were opened and adult thrips and nymphs extracted by washing in 70% alcohol. All the thrips were then sorted, identified, counted and recorded. Identification was done using characteristics such as color, the rows of setae along the veins of the fore wings, the number of antennal segments and the positioning of the pair of setae III on the imaginary triangle of the occelli (Ralph, 1998; Stiller, 2001).

All the thrips species from the plant parts collected from the set of 4 plants or the quadrat were sorted according to their characteristics, identified, counted and recorded. The data was then analyzed using Genstat Statistical Software, version 14. To determine if the infestation levels of thrips on *D. lablab* differed significantly between irrigated and rain fed *D. lablab, ANOVA* was performed on transformed count data. *Post hoc* test was done using the Student-Neuman-Keuls test. The rejection level was set at $\alpha < 0.05$.

RESULTS

Thrips species in irrigated and rain-fed *D. lablab* in Meru sub-county were dominated by *Megalurothrips sjostedti* during both dry and rainy seasons. During the dry season, adults of *M. sjostedti* infesting *D. lablab* were

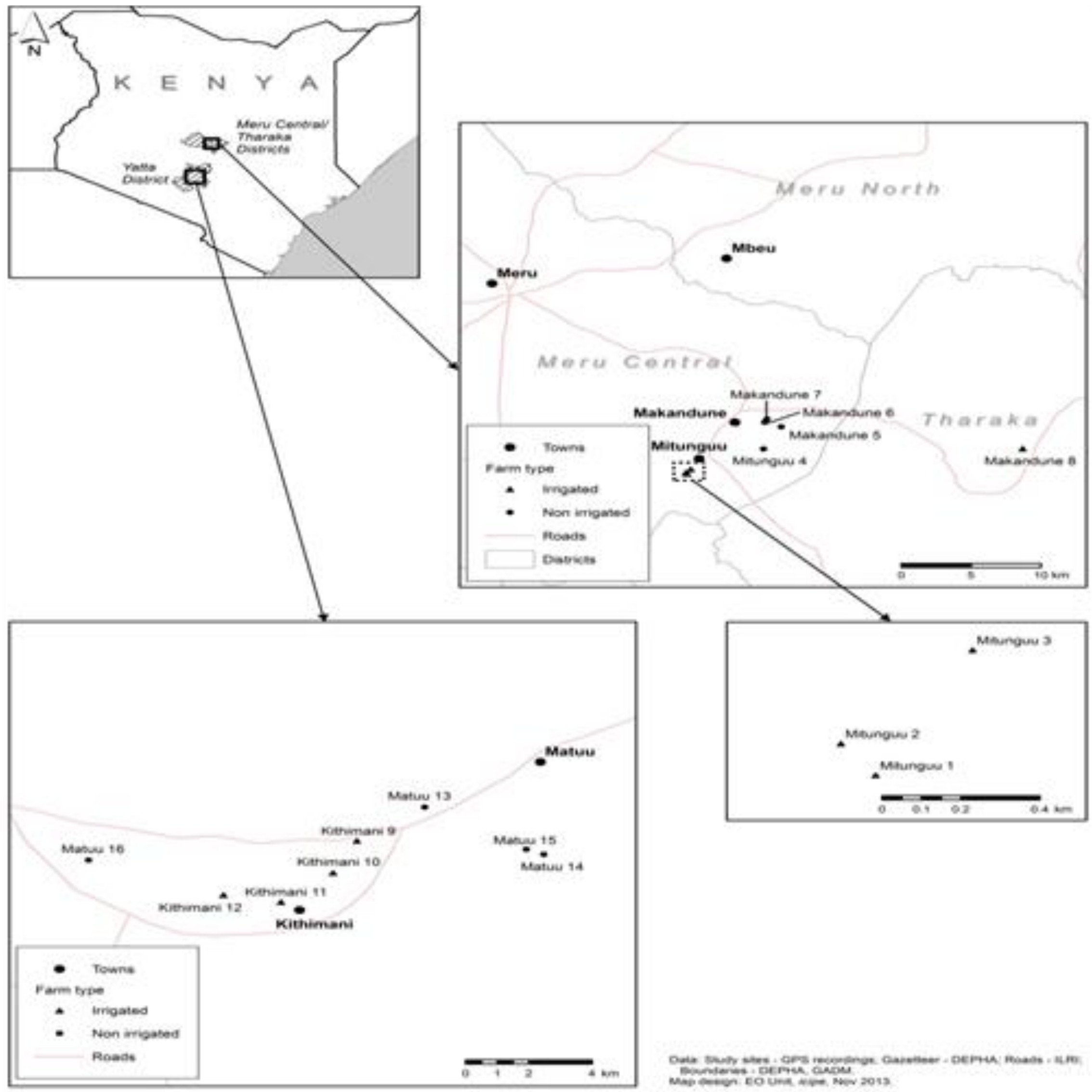

Figure 1. Location of the study sites in Meru Central and Yatta Sub-counties, Kenya.

significantly higher ($F_{(3,57)}$=4.73; $p<0.05$) on irrigated farms with a mean number of 176.2±11.0 as compared to 117.1±3.0 on rain fed farms (Table 1). Infestations were also significantly higher in irrigated farms ($F_{(3,71)}$=8.41; $p<0.05$) during the wet season with a mean of 109.4±10.8 as compared to 19.6±7.9 thrips on rain-fed farms (Table 2).

In Yatta Sub-county, however, rain-fed *D. lablab* had a significantly higher number of adult *M. sjostedti* ($F_{(3,57)}$=4.73; $p<0.05$), averaging 117.2±18.3 as compared to 59.6±12.2 in irrigated *D. lablab* during the dry season (Table 1). Similarly, during the rainy season, infestation by adult *M. sjostedti* was also significantly higher in the rain-fed ($F_{(3,71)}$=8.41; $p<0.05$) than the irrigated *D. lablab*, averaging 114.3±10.2 as compared to 141.7±11.0 in the rainy and irrigated farms, respectively (Table 2).

Other species of thrips commonly infesting *D. lablab* in Meru and Yatta Sub-counties were *Frankliniella occidentallis* (Pergade), *Frankliniella schultezi* (Pergade) and *Hydatothrips* spp. The infestation levels of adult *F. occidentallis* in Meru sub-county was highest in irrigated *D. lablab* during the dry season, with a mean of 10.7±2.0, which was significantly higher ($F_{(3,57)}$=3.15; $p<0.05$) than rain-fed *D. lablab* 2.6±1.4 (Table 1). During the wet season, infestation levels of adult *F. occidentallis* were similarly significantly higher ($F_{(3,54)}$=0.88; $p<0.05$) in the irrigated *D. lablab*, averaging 30.6±4.3 as compared to 23.7±5.1 in rain-fed *D. lablab.*

In contrast, infestation rates by *F. occidentallis* in Yatta Sub-county were lower in the irrigated than in the rain fed *D. lablab* during the dry season (Table 1). However,

Table 1. Means (±SE) of counts of various thrips species in irrigated (vegetable type) and rain fed (grain type) conditions in Meru central and Yatta sub-counties (Dry season). Means with the same letter within the same row are not significantly different at α<0.05. Means separated using SNK test.

Thrips species	Means (±SE) of counts per m²			
	Meru		Yatta	
	Irrigated (vegetable type)	Rainfed (grain type)	Irrigated (vegetable type)	Rainfed (grain type)
M. sjostedti	176.2±11.0^{c}	117.1±3.0^{b}	59.6±12.2^{a}	117.2±18.3^{c}
F.occidentallis	10.7±2.0^{b}	2.6±1.4^{a}	1.3±1.1^{a}	3.7±2.2a^{b}
F. schultzei	10.0±2.0^{b}	7.4±2.6^{ab}	4.1±2.2^{a}	5.6±3.3^{ab}
Hydatothrips spp.	4.7±1.6^{a}	9.0±2.0^{b}	3.3±1.8^{a}	2.7±2.0^{a}

Table 2. Means (±SE) of counts of various thrips species in irrigated (vegetable type) and rain fed (grain type) conditions in Meru central and sub-counties Yatta (Wet season). Means with the same letter within the same row are not significantly different at p<0.05. Means separated using SNK test.

Thrips species	Means (±SE) of counts per m²			
	Meru		Yatta	
	Irrigated (vegetable type)	Rain fed (grain type)	Irrigated (vegetable type)	Rain fed (grain type)
M. sjostedti	109.4±10.8^{b}	19.6±7.9^{a}	141.7±11.0^{d}	114.3±10.2^{c}
F. occidentallis	30.6±4.3^{b}	23.7±5.1^{ab}	15.9±4.3^{ab}	8.9±4.1^{a}
F. schultzei	7.8±2.0^{a}	10.0±2.3^{a}	5.7±2.0^{a}	3.1±1.9^{a}
Hydatothrips spp.	2.9±2.4^{a}	9.7±3.0^{b}	3.8±2.6^{a}	10.9±2.3^{b}

during the wet season, infestation rates by *F. occidentallis* were significantly higher in irrigated than in rain-fed *D. lablab* (Table 2). During the dry season, infestation level of *Hydatothrips* spp. was significantly higher in rain fed *D. lablab* in Meru, with adult mean numbers of 9.0±2.0 compared to irrigated *D. lablab* in both sub-counties (Table 1). In wet season, the highest infestation of *Hydatothrips* spp. was in rain fed *D. lablab* in both Meru and Yatta with adult mean numbers of 9.7±3.0 and 10.9±2.3 respectively. These means were not significantly different ($F_{(3,71)}$=1.75; p>0.05) from each other. However, they were significantly higher ($F_{(3,71)}$=1.75; p<0.05) than irrigated *D. lablab* in both Meru and Yatta (2.9±2.4 and 3.8±2.6) (Table 2).

DISCUSSION

From the results, thrips infestation levels were significantly higher in irrigated *D. lablab* compared to rain fed in Meru. Irrigation provided good growing conditions for the *D. lablab* plants to ensure rapid growth hence enough shoots and flowers for thrips to feed on. This is in line with the findings of Kasina et al. (2009) who found that flower thrips infestation was high in the shoots and flowers compared to other parts of the crop. In *D. lablab*, irrigation brings about longer growing seasons which enables these species of thrips to complete a greater number of reproductive cycles during the growing season (Kannan and Mohammed, 2001).

However, environmental stress such as drought weakens plants and makes them more susceptible to thrips attack. This explains why the *M. sjostedti* infestation levels were higher in rain fed compared to irrigated in Yatta. This was observed by Gitonga (1999) in French beans who reported that plants under water stress are very susceptible to direct thrips damage. *Megalurothrips sjostedti* is widely spread in sub Saharan Africa and highly polyphagous in the family *leguminosea* (Seif *et al.,* 2001). This suggests why *M. sjostedti* was more abundant than any other thrip species in this study.

Conclusion

Infestation levels of the species of thrips attacking *D. lablab* in Meru central and Yatta sub-counties of eastern region of Kenya varied significantly between irrigated and rain fed conditions. *Megalurothrips sjostedti, F. occidentallis* and *F. schultzei* infestation levels were significantly higher in irrigated *D. lablab* (vegetable type) than rain fed *D lablab* (grain type) in Meru central. This was extremely different in Yatta where *M. sjostedti* infestation levels were significantly higher in rain fed than irrigated.

Hydatothrips spp. infestation levels were significantly higher in rain fed *D. lablab* compared to irrigated *D. lablab* in both sub - counties*.*

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Conflict of Interests

The authors have not declared any conflict of interests.

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Evaluation of biological control agent *Pantoea agglomerans P10c* against fire blight in Morocco

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The bacterial antagonist *Pantoea agglomerans P10c* was evaluated for efficacy to control fire blight in Morocco. It was evaluated in the laboratory on detached blossoms (pre and post-inoculation of pathogen) and under field for its ability to suppress growth of *Erwinia amylovora* on blossoms of pear trees (*Pyrus communis*); and for its aptitude to establish on and to colonize the blossoms of apple tree (*Malus domestica*) under field conditions. Results revealed that the installation of *P. agglomerans* P10c strain on detached blossom before the inoculation with the *E. amylovora* (preventive treatment) allows an efficient control of the disease (96%), with an incidence that never exceed the level 2.5%. but for curative treatment the incidence increased to 40% and the efficiency was only about 32%. Under field, *P. agglomerans* P10c applied a twice (10 and 60% bloom time) at 10^8 CFU/ml reduced significantly the incidence of fire blight by 64.7 to 76.9%. The experiment of the establishment and the survivability of *P. agglomerans* P10c in the stigma showed a positive evolution which increased from 4.5×10^4 to 7.6×10^5 CFU/flower. Results from this work illustrate that this antagonistic strain is able to control efficiently fire blight in apple and pear trees under the conditions of the arias of Meknes.

Keys words: *Erwinia amylovora*, *Pantoea agglomerans* P10c, antagonist, blossom, preventive treatment, apple tree, pear tree, Meknes.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill, 1883), is a devastating disease of rosaceous plants (Van der Zwet, 1979). This plant pathogen has been reported from several regions of the world including North and Central America, New Zealand, United Kingdom, Europe, Middle East and Central Asia (Smits et al., 2010). The disease was introduced in Morocco in 2006, in a commercial orchard in the rural area of Ain Orma (Saoud, 2008), and it has spread rapidly throughout the most important pome fruit production regions causing significant and serious economic losses (Fatmi, 2009; Yaich et al., 2011). Fire blight can affect all

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aerial parts of the plant, but blossoms are the most important site of infection for epidemic blossom blight (Norelli et al., 2003). Therefore, the suppression of the infection during blossom stage is the pillar of the strategy to control the disease. In addition, the pathogen needs to multiply on the stigmatic surfaces before penetrating and infect the flower (Johnson and Stockwell, 1998). Indeed, if the multiplication of *E.amylovora* is inhibited, the probability of the infection of the blossom and the dispersion of the bacteria to other blossoms will be reduced. The elimination of the pathogen in this period couldn't be ensured by the chemical products currently registered in Morocco against fire blight because of their adverse phytotoxic effects on blossoms and fruits. Whereas, the use of antagonists of *E. amylovora*, such as *P. agglomerans* (Stockwell et al., 2002; Vanneste, 2011) and *Pseudomonas fluorescens* (Cabrefiga et al., 2007, 2014), has been widely demonstrated as an alternative for the control of fire blight in several countries. The objective of this study was to test the efficacy of a product based on the strain *P. agglomerans* P10c under laboratory and field conditions in the country of Morocco.

MATERIALS AND METHODS

Bacterial strains

The bacteria used in this study were the bacterium *P. agglomerans P10c*, approved in Italy under the name of PomaVita® (10^{11} CFU/g) and in New Zealand under the name of Blossom Bless®. This antagonistic bacterium is resistant to streptomycin (100 ppm) and rifampicin (20 ppm). *P. agglomerans P10c* was supplied in lyophilized form, the identity and purity of strain was verified by biochemical tests using API 20 E system after 10 serial dilution plating on LB agar of the suspension of 1 g of PomaVita in 1 ml of sterilized water. The pathogen *E. amylovora* was isolated from a commercial pear orchard (cv Cossia), located in the region of Meknes, referring to the EPPO Bulletin (2004). The identity of *E. amylovora* was checked by amplification of 1 kb fragment of the plasmid pEA29 by the PCR technique (Bereswill et al., 1992). Thereafter, the *E. amylovora* quantity was increased using the Levane culture medium at a temperature of 25°C.

Detached blossoms assay

Evaluation of the efficacy of *P. agglomerans P10c* was conducted in laboratory conditions on detached blossoms pear cv. Duke of Bordeaux. blossoms were collected at the white button stage from pear orchard (planting 2004) in the region of Meknes, putted in plastic bags on ice, and transported to the laboratory. In the laboratory, collected flowers were maintained in submersion in water contained in 2 ml tubes. Tubes containing water were supported by a polyester rack and each rack contains 20 to 24 blossoms constituting the experimental unit. The later were placed in a growth chamber (2.5 m length, 1.5 m in width and 1 m in height). The temperature, humidity and lighting were: 22°C day / 10°C night, 90 ± 10% and 17 h, respectively (Wilson et al., 1992). Treatments were assigned to 20 newly opened blossoms per treatment in a randomized complete block design with four replications. The blossoms were grouped into blocks, based on their availability in the field. The total amount of flowers was 320 (20 × 4 × 4). In order to simulate appropriate conditions to test the effectiveness of *P. agglomerans P10c*, four treatments were applied: (1) *E. amylovora* alone as a control (E0); (2) *P. agglomerans P10c* before *E. amylovora*: Pre-inoculation (PE); (3) *E. amylovora* then *P.agglomerans P10c*: Post-inoculation (EP) (4) *P. agglomerans P10c* alone (P). The antagonist was prepared by diluting 1 g of the lyophilized product in 1 L of distilled water to produce recommended doses and it was applied on blossoms using a micro-sprayer. 24 h later, the pathogen was suspended at a concentration of 5×10^8 CFU / ml and was applied to flowers using a brush. The concentration was adjusted using an optical density reader at 600 nm after preparation of the suspension across 5 Mac Ferland. After four days of pathogen inoculation, daily tracking for a period of 5 days of the number of blossoms, at each treatment, showing symptoms of fire blight was performed. To compare the effect of different treatments, the area under the curve progression disease (AUDPC) was calculated using the formula of Shaner and Finney (1977):

$$\mathbf{AUDPC} = \sum_{i=1}^{n} \left[\frac{y_i + y_{i-1}}{2}\right] \times (t_i - t_{i-1})$$

yi is the incidence of the control disease for treatment i at times t and n the number of reading dates incidence.

The effectiveness of the two treatments: *P. agglomerans P10c* then *E. amylovora* and *E. amylovora* then *P. agglomerans P10c* was calculated relative to the control at the end of the trial by the following formula:

$$E = (y_0 - y_i)/y_0$$

With *y0* the incidence of the control and *yi* the incidence of the treatment *i.*

Biological control of fire blight on pear trees

The trial was conducted in a commercial orchard of pear variety Cossia planted in 2004, it is located Toulale in the region of Meknes. The antagonist was prepared referring to the instructions contained in the data sheet of the commercial product (10 g of the lyophilized product in 10 L of water, that is, 10^8 CFU/ml). The application on the trees was carried out using a knapsack sprayer with pressure maintained until runoff. Two applications of the antagonist was performed (Stockwell et al., 2010), one at 10% bloom time and the second a week later (60% bloom time) under conditioned temperature 20°C and low wind. Treatments were assigned to a tree by treatment in a completely randomized design with five replicates. The trial was carried out under natural inoculation. For the evaluation of treatment, the disease incidence on blossoms was calculated. It was measured by calculating the number of blighted blossoms relative to the total number blossoms of observed branch. Ten branches were randomly observed for each tree, in total 50 branches considered by treatment with an average of 30 blossoms per branch. This evaluation of was carried out one week after the second application of the antagonist.

Establishment and survival of P. *agglomerans* P10c on apple flowers under field conditions

The trial was conducted over a period of two weeks of blooming period, on apple trees (cv. Golden). To test the establishment and survival of *P. agglomerans P10c* on apple blossoms under field conditions, newly opened blossoms (less than 24 h) were selected and marked by a plastic string. The marked blossoms were randomized in two identical blocks to have 80 blossoms per block, with additional blossoms to prevent the risk of loss by wind or insects. In total, 200 blossoms were marked. Flowers of one of this blocks were sprayed with a suspension of *P. agglomerans* P10c using a micro-sprayer. The suspension was prepared by diluting 1 g of the lyophilized product in 1 L of distilled water. In parallel, the group of blossoms of the other block was sprayed with water only. These blossoms were considered as control. Weather conditions were monitored using a thermo-hygrograph in the shadow, placed 2 m from the ground. After spraying, 12 flowers per block were taken every day for 7 days. The 8^{th} and 9^{th} sampling were done at a rate of 3 days after the 7th sampling. The collected flowers were transported to the laboratory in small and sterilized bags. Analysis and determination of *P agglomerans* P10c population were done according to the following protocol (Francesco et al., 2011): Under the laminar flow hood, each blossom was treated individually; the five stigmas were removed using sterile forceps and were washed in 1 ml of 10 mM MgSO4. Washing was done in an Eppendorf tube with vortexing for a few seconds. Solutions were subject to a series of dilutions of 1:10 th in 10 mM $MgSO_4$. (that is, 100 µl of lavat in 900 µl of 10 mM $MgSO_4$). 50 ml of each dilution was plated on LB agar supplemented with streptomycin (100 ppm) and rifampicin (20 ppm). For the blossoms treated with water, plating of the solutions was performed directly without dilution. Petri dishes were incubated at a temperature of 25°C and colony counting was made after 24 h; the bacterial concentration by CFU/ml was obtained by multiplying the number of CFU/Petri dish by 1000 ml/50 ml. Finally, the CFU/blossom concentration was obtained by calculating the initial concentration.

Statistical analysis

The calculated efficiencies was transformed using arcsin√. The data obtained, AUDPC and arcsin√E, were analyzed by ANOVA. The Student-Newmen-Keuls test was used to separate means for significant effect at the 0.05 probability level. All statistical analyses were performed with SPSS 17 (IBM) software.

RESULTS

Detached blossoms assay

The fire blight symptoms were observed in the control treatment. The average number of infected flowers varied during the test period. Indeed, four days after inoculation, the average incidence of the disease on the affected flowers is about 40% for the control (E0) and 20% for the treatment (EP). This incidence increased to a maximum of 60% for the control and 40% for the treatment EP. Furthermore, preventive treatment (PE) expressed an incidence that does not exceed the threshold of 2.5% during the entire test period (Figure 1). Analysis of variance of AUDPC shows a highly significant effect between treatments and the comparison of means by Student-Newmen-Keuls test ($p < 0.05$) showed the presence of three classes as indicated in Table 1.

In terms of efficiency, treating flowers with *P. agglomerans* P10c preventively significantly reduced the incidence of the disease by about 96%. This efficiency was only about 32% in the curative cases.

Biological control of fire blight on pear trees

The incidence of blossom infection in control pear trees naturally inoculated with the pathogen was high (65.7% in a trial 1 and 72% in a trial 2). The performance of P10c for disease control varied insignificantly among trials (Figure 2). In trial conducted in 2013, *P. agglomerans* P10c significantly reduced the percentage of blighted blossoms on commercial pear orchard by 64.7% compared to water-treated control. In trial conducted in 2015, this percentage of reduction increased to 76.9%.

Establishment and survival of *P. agglomerans* P10c on apple flowers under field conditions

In all samples (for 9 days), *P. agglomerans P10c* was isolated from flowers inoculated by spraying. The isolation success rate at the first sample (D0 + 1) was above 90%. This rate was generally maintained throughout the test period. The size of the populations of *P. agglomerans P10c* isolated from flowers shows a growing tendency to change over time ranging between 10^4 and 10^8 CFU/flower suggesting multiplying of the antagonistic bacteria on the flower surface (Figure 3). Indeed, the initial size of the population of *P. agglomerans P10c* was estimated between $1×10^4$ and $1×10^5$CFU/flower. Subsequently, the population has grown to stabilize around $5.5×10^5$ CFU/ flower during the three days. Then, a slight decrease was noticed and that is mainly due to the recording night temperatures below 10°C , then the multiplication of the bacteria took over eventually reaching $7.6×10^5$ CFU / flower. In terms of non-inoculated flowers (control), no colonies of *P. agglomerans P10c* was obtained on the culture media during the entire test period.

To explain the influence of climatic conditions on the biotic potential of *P. agglomerans P10c*, correlations between key climate parameters and population levels of the antagonist were established (Figure 4). A positive correlation between daily mean temperature and the average size of the established population was observed. A strong influence of temperature on the development and survival of *P. agglomerans* P10c was recorded. For cons, the minimum relative humidity was negatively correlated with the studied parameter.

DISCUSSION

To be effective, the biological control agent should be

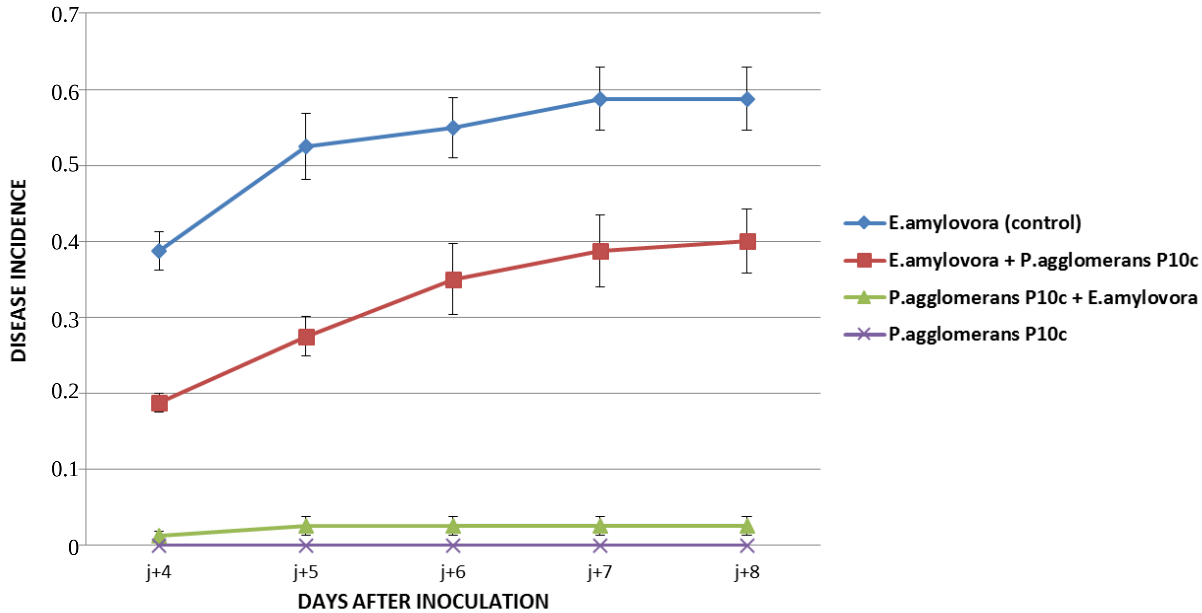

Figure 1. Evolution of the average incidence of symptoms of *E. amylovora* inoculated to pear blossoms cv. Duke of Bordeaux before or after de application of P10c at 10^8 CFU/ml under laboratory conditions (growth chamber [2.5 m length, 1.5 m in width and 1 m in height]. The temperature, humidity and lighting were 22°C day/10°C night, 90 ± 10% and 17 h, respectively).

Table 1. Area under the curve progression disease (AUDPC) of different treatments (detached blossoms assay).

Traitement	AUDPC
E. amylovora (E0)	2.25[a]
E. amylovora than *P. agglomerans P10c* (EP)	1.25[b]
P. agglomerans P10c than *E. amylovora* (PE)	0.12[c]
P. agglomerans P10c (P)	0.00[c]

Treatments followed by the same letter are not significantly different according to the least significant difference test at the 0.05 level.

able to multiply at the same ecological niche of the pathogen. This results in competition on the space and nutrients. It was determined that the competition on the sites and on the nutritional substrates limiting growth is the main mechanisms by which *P. agglomerans* suppresses the growth of *E. amylovora* on the stigma (Özaktan and Bora, 2004). This explains the result obtained when *P. agglomerans* P10c was applied to flowers before infection with the pathogen (preventive treatment). Otherwise, the result of curative treatment could be explained by mechanisms other than competition on the ecological niche. Wodzinski et al. (1994) report that *P. agglomerans* inhibits *E. amylovora* by acidification of the medium, producing bacteriostatic substances or bacteriocin herbicolin or induction of hypersensitivity reactions. Stokwell et al. (2002) show that *P. agglomerans* has a capacity to produce antibiotic contributing to the suppression of fire blight.

Under field conditions, Johnson et al. (1993) found that early establishment of populations exceeding 10^5 CFU per blossom of *P. fluorescens* Pf A-506 and *P. agglomerans* Eh C9-1 on pear blossoms suppressed establishment and growth of *E. amylovora*. The incidence of fire blight on blossoms was reduced by about 60% with two applications of bacterial antagonists in experimental plots in the Pacific Northwest (Nuclo et al., 1996) and California (Lindow et al., 1996).

The population level may be influenced by temperature and humidity, our results are agree with those obtained by Johnson et al. (2000) and Stockwell et al. (2002) for *P. agglomerans* strain Eh252 under the conditions of the State of Oregon (USA). Finally, to be effective, the antagonistic bacteria must settle the entire stigmatic surface in the orchard conditions (Johnson et al., 1993; Lindow and Suslow, 2003), and the size of the population of the antagonist on the stigma must be between 10^5 and 10^6 CFU / flower (Wilson et al., 1992; Wilson and Lindow, 1993). In this study, detectable average population size of *P. agglomerans* P10c ranged from 4.5×10^4 to 7.6×10^5 CFU / flower under real conditions during the 12 days after the date of inoculation (Figure 3). This result shows that *P. agglomerans* P10c has colonized the flowers in the real conditions and could contribute significantly to reduce the *E. amylovora* installation.

In conclusion, the installation of the strain *P.*

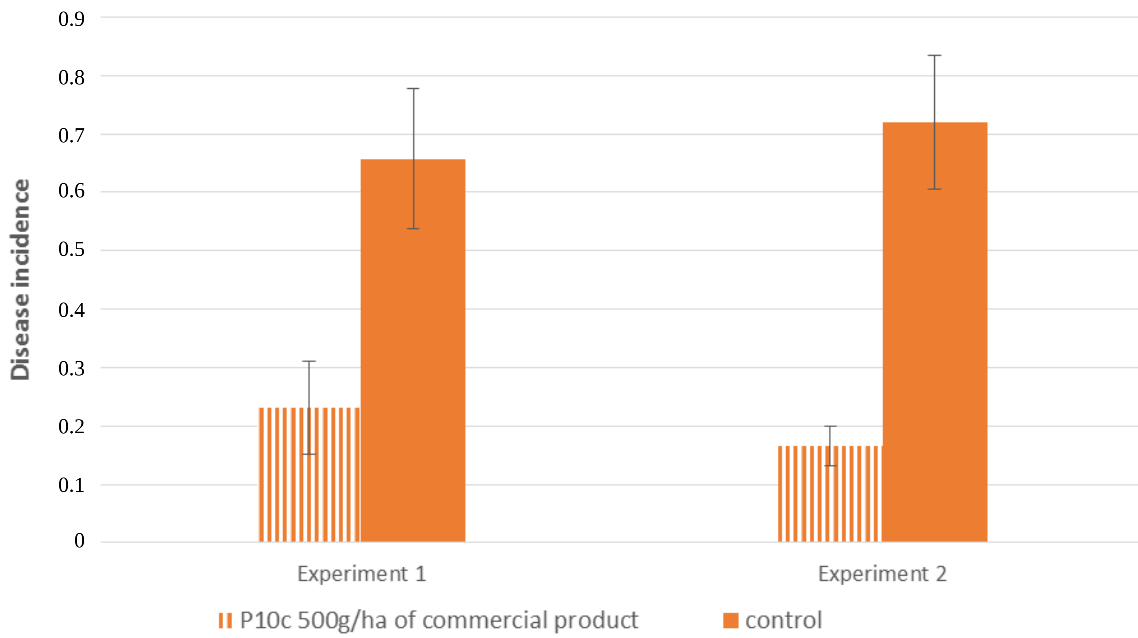

Figure 2. Effect of Antagonist strain P10c on the occurrence of fire blight on pear blossoms one week after the second application of the antagonist. Tow application of 500 g/ha of commercial product were applied at 10 and 60% bloom time (Experiment 1 in 2013, Experiment 2 in 2015)

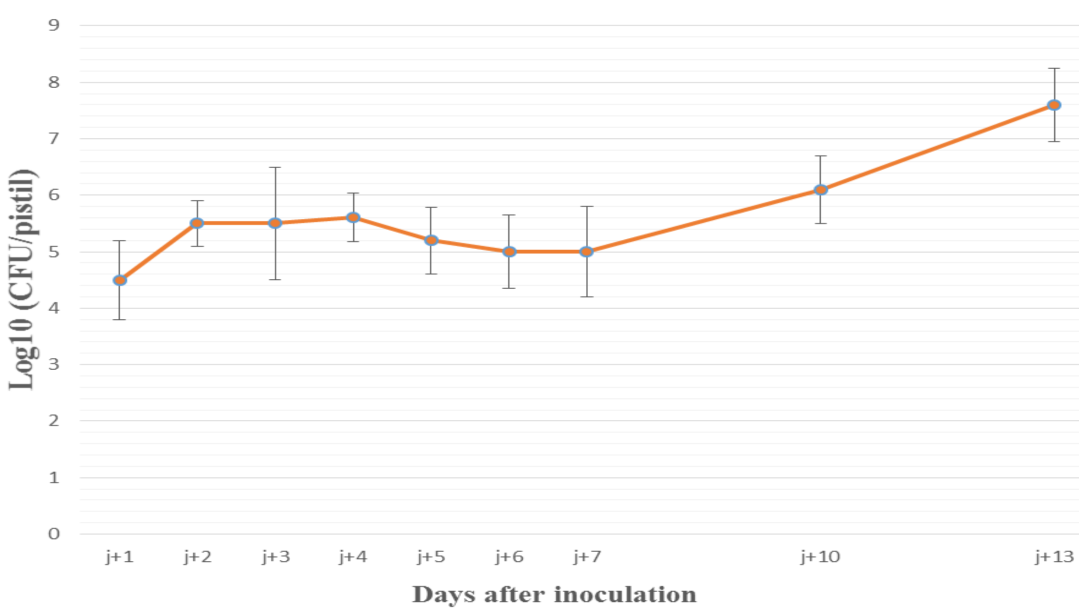

Figure 3. Mean population level of *P. agglomerans P10c* under real conditions on apple blossoms cv. Golden.

agglomerans P10c before inoculation with the pathogen has significantly slow the disease. Under field conditions, the monitoring of its dynamics shows an increasing tendency to evolution indicating the reproduction of this antagonist on the flower surface. The estimated size of the population of *P. agglomerans P10c* on the blossoms and the efficacy trials shows that this antagonistic strain would be able to control effectively fire blight in the

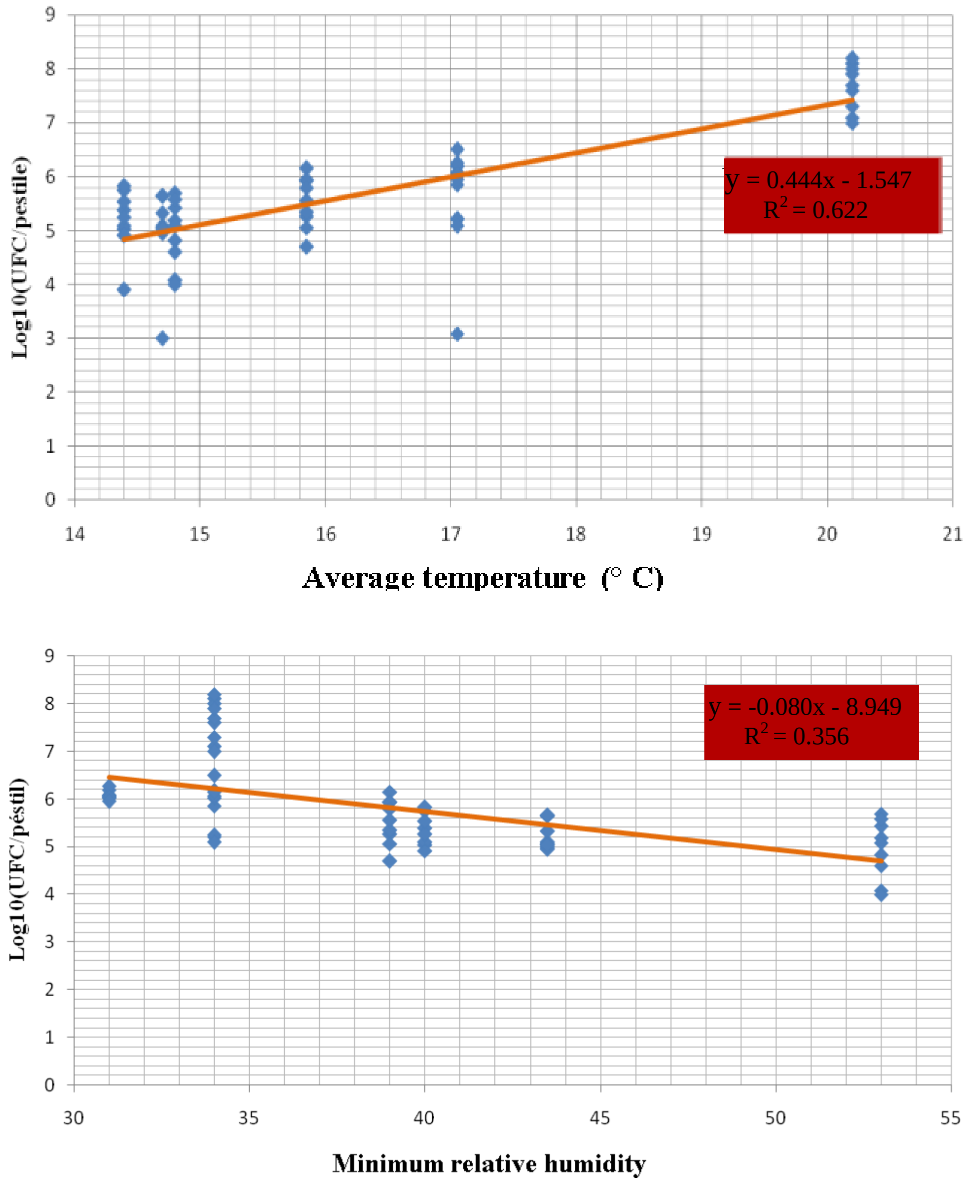

Figure 4. Correlations between population levels of *P. agglomerans* P10c and the average temperature (top), relative humidity (below).

orchard conditions in Meknes region.

Conflict of Interests

The authors have not declared any conflict of interests.

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