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Review article

THE MOST IMPORTANT DILEMMAS REGARDING THE WELFARE OF FARM ANIMALS

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Abstract: In this review paper, contradictions in modern livestock production as dilemmas of farm animal welfare are considered. The main dilemma concerns the question of whether extensive production in small farms is generally better than intensive production in large farms. The next dilemma relates to an intensive selection of animals and its impact on the emergence of welfare problems. Another dilemma is related to the two main interconnected problems of pig welfare in individual farrowing pens: the piglet death by crushing and the sows' movement restriction. Similarly, welfare dilemma is the paradox of parent flocks of broiler line breeding that could not be solved until the pressure for genetic advancement in production is required due to the economic efficiency. The next example of the dilemma is the widespread practice of tail docking in piglets in order to reduce the risk of tail biting. Although the tail docking is painful and may cause death, anaesthesia is usually not applied on farms. A similar example of the dilemma is debeaking in the laying hens and the occurrence of feather pecking in free rearing systems. It is important to notice the difference between the described dilemmas, where one premise opposes the other, which is essentially a conflict between the animal welfare goals and other values, such as economic, ethical and moral issues. Finally, there are also some dilemmas about the consumers' willingness to pay a higher price of products that originate from welfare friendly rearing conditions.

Key words: dilemma, welfare protection, farm animals.

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Introduction

There are numerous contradictions in contemporary livestock production, which are now considered in the literature as dilemmas in terms of ensuring the welfare of farm animals (Appleby et al., 2014). These dilemmas concern equally all important participants in the technological process of farm production, consumers of animal products and the public as a whole (Hristov et al., 2007a). The important participants in the technological process of production related to this issue are farm owners, employees, stockmen, veterinarians, animal husbandry engineers and advisers, animal feed suppliers, technical and other persons who participate directly or indirectly in any stage of farm production (EFSA, 2012c). In addition, the dilemmas are also important for the activists of NGOs as well as governmental institutions that define and adopt appropriate regulations for ensuring good welfare of farm animals (Kjarnes et al., 2007; Hristov et al., 2007b).

The issue of animal welfare on farms was initiated 65 years ago because of public concern about the problems that arose as a result of increasing the capacity of the farm, mass keeping of animals in one place and industrialization of farm production. Use of the term factory animal production (Harrison, 1964) symbolically represents a refusal of the public to accept industrial intensive system due to the perception that this farm production system is not able to provide the appropriate environmental conditions for the animals (Brambell, 1965; Miele at al., 2011). Many people today perceive that animal welfare is significantly better in small capacity production units and open farm systems with a natural diet. They reject husbandry practices that are associated with industrial production systems as poorer regarding animal welfare, human health and protection of the environment (Cornish et al., 2016).

On the contrary, poor evidence of any clear relationship, negative or positive, between farm capacity and animal welfare was found (Robbins et al., 2016), indicating that larger farms can achieve more in animal welfare improvement, but may create welfare risks as well. Today, the need to update thoughts on animal welfare in the sense that it moves away from the “Five Freedom” to “Life worth living” is often pointed out (Mellor, 2016). In the papers published by von Keyserlingk et al. (2009) and Nawroth et al. (2019), it has been noted that general knowledge about farm animals’ relations with the environment is necessary, and it has significant importance for a range of stakeholders (citizens, politicians, cognitive ethologists and philosophers).

In addition to those resulting from inadequacy in housing conditions, many other factors can also affect the welfare of animals, particularly of farm animals reared in industrial production systems, such as: selection for high productivity, restricted nutrition, high-density population, poor zootechnical procedures and veterinary treatments which often inflict stress, pain and occasionally suffering to

animals (Vučinić, 2006; Broom and Fraser, 2007). Also, management, disease preventive measures, handling of animals, animal and space hygiene, rearing conditions such as floor and the manure system influence significantly disease outbreaks and provide a good welfare level for farm animals (OIE, 2018a). Many of the proposed methods for addressing these issues of animal welfare ensured in industrial production systems include compromise, but in some cases solving one problem can lead to other problems, which can be even much more pronounced. These issues, in general, are considered as welfare dilemmas in recent literature (Appleby et al., 2014; Hötzel, 2014).

In making professional decisions, veterinarians, animal husbandry engineers and stockmen have to be guided by their conscience, adherence to ethical behaviour, professional guidelines, cultural norms and legal framework. Therefore, it is crucial to define and establish good farming practices through professional ethical guidelines. Since farm animal welfare dilemmas are very important for professional ethical guidelines, the aim of this paper is to identify and discuss the most important ones.

The most important animal welfare dilemmas

A large number of citizens in developed countries estimate current conditions in livestock production operations as insufficient in respect of farm animal welfare. There are certain controversies that contribute to maintaining a poor state of farm animal welfare. Consumer behaviour is often contradictory; they verbally support the improvement of farm animal welfare but are not ready to pay higher price for animal products derived from animal welfare friendly livestock production (e.g. Boulstridge and Carrigan, 2000; Tawse, 2010), which is often referred as citizen-consumer duality (Vanhonacker et al., 2007).

All the most important criticisms of intensive farming systems are the result of the events and changes that these systems have undergone in the last two decades of the 20th century. In intensive rearing systems, the mortality of animals caused by parasites and by the pathogens, as well as by predation and bad weather conditions, has been reduced. However, mortality from respiratory, gastrointestinal organs and locomotor system, as well as behavioural disorders, has increased (Vučinić, 2006; Broom and Fraser, 2007; Hristov et al., 2007b, OIE, 2018a). Also, intensive rearing systems brought with them very important problems related to direct pollution of the environment (McGlone, 2001; Vučinić, 2006).

It is very important to point out that there is no animal rearing system that can provide or replace living conditions in autochthonous habitats. Intensive systems of animal rearing regularly deprive them of sunlight, fresh air, physical activity and expression of normal forms of behaviour. In confined rearing systems, there is the possibility of better control over animals, early detection of disease and

maintenance of better hygienic conditions as a whole, but animals are predisposed to the more frequent occurrence of infectious diseases, which requires either immune prophylaxis or antibiotic treatment. Contrary to confined systems, open free systems of rearing theoretically allow animals to express physiological forms of behaviour, but in these systems, animals are constantly exposed to parasitic diseases, bad weather conditions and predators. Regardless of all open animal rearing system shortcomings, greater immune competence and the ability to express physiological forms of behaviour give them an advantage over confined systems of rearing (Figure 1). Therefore, in many countries, the reorientation of breeders from intensive confined to open free systems of animal rearing followed (Hristov et al., 2006c; Vučinić, 2006; Broom and Fraser, 2007; Hristov et al., 2007a).

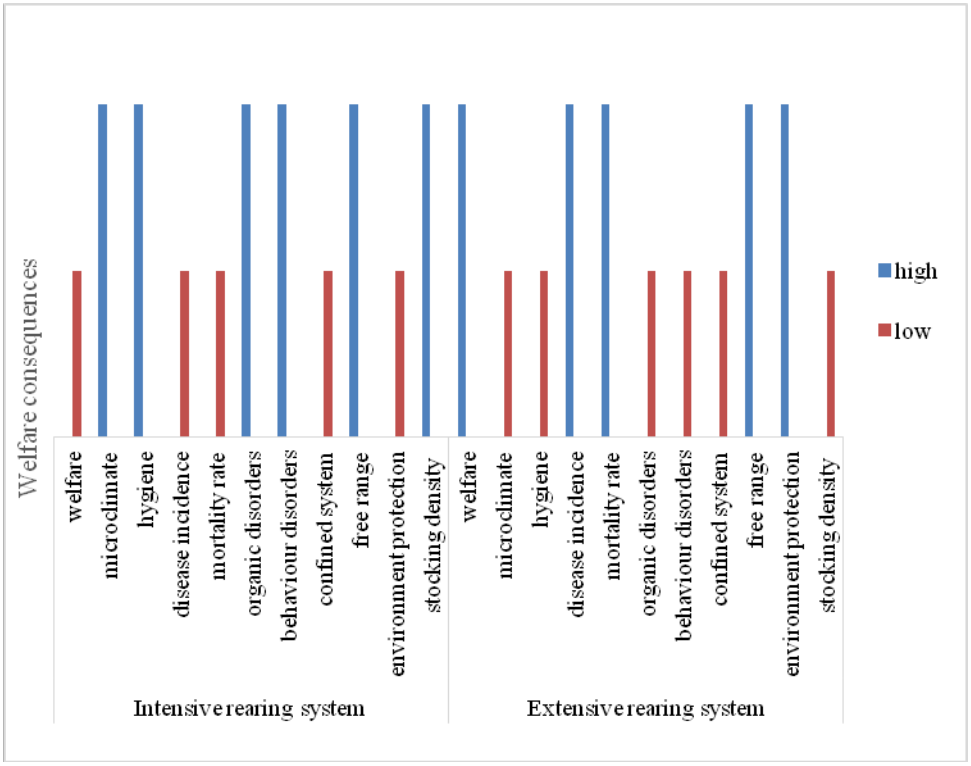

Figure 1. Welfare consequences in different rearing systems.

The knowledge of physiological aspects of behaviour and welfare (Todorović-Joksimović et al., 2007), emotions and cognition (Fratrić et al., 2007) and stress and welfare of farm animals (Hristov et al., 2007c) contributed to the definition of

minimum standards for ensuring the welfare of farm animals (Hristov et al., 2007d, e; Pandurović et al., 2007; Petrović et al., 2007). The Office International Epizootique (OIE) has so far adopted several chapters related to the welfare aspects of farm animal production systems, including pig (OIE, 2018b), dairy cattle (OIE, 2018c), beef cattle (OIE, 2018d) and broiler chickens (OIE, 2018e), which continue to contribute to the adoption of welfare standards for farm animals in many countries, especially developing ones.

In the last 65 years, understanding of higher cognitive processes in animals advanced dramatically (Nawroth et al., 2019), as well as in the development and validation of farm animal welfare assessment methods (EFSA, 2012a, b, c, d, e, f; EFSA, 2015a, b, c). Perhaps the most significant improvement of the welfare of farm animals was achieved in the conditions of housing with the adoption of the five freedoms proposed by the Brambell Committee: to be able to get up, lie down, turn around, do self-care and stretch their limbs. The conditions of housing preventing animals from the abovementioned freedoms are gradually abandoned in certain parts of the world, by legislation or guidelines, implementation of welfare programs or farm assurance schemes (Main et al., 2014). Further visible progress has been made with the implementation of research on welfare indicators, i.e. resource-based, management-based, and especially, animal-based indicators (Bartussek et al., 2000; Botreau et al., 2007; Blokhuis, 2008; Welfare Quality, 2009a, b, c; Hristov et al., 2009; Mellor, 2016).

Genetic selection is one of the main drivers of the increased production of farm animals. This raises profits but also amplifies direct and indirect losses in animals. Therefore, some authors point out that the selection for high production imposes many animal welfare problems (Figure 2), especially in industrial livestock production (Rodenburg and Turner, 2012; Grandin and Deesing, 2014). Intensive selection towards high production has led to the frequent occurrence of numerous diseases in farm animals. For example, high-milk dairy cows are prone to mastitis, lameness, milk fever, placental retention, ketosis, endometritis and occurrence of cysts on the ovaries (EFSA, 2009a, b; Oltenacu and Broom, 2010). In addition, the selection of growing production may cause morphological and physiological imbalances in pigs, resulting in the occurrence of locomotor disorders and the reduction of the adaptive ability of animals to environmental challenges. Increasing the average size of the litter by selection resulted in higher mortality of the piglets. A general consent was achieved that these can reduce animal welfare towards starvation, pain or discomfort, and in some cases impede the reproduction and longevity of pigs (EFSA, 2005a, 2007a, b). Egg production, for example, affects the cardiovascular and musculoskeletal system in the laying hens (EFSA, 2005b). Although it was possible to expect, otherwise, initiatives and attempts to reconsider the objectives of productivity selection in terms of incorporating features that could favour animal welfare benefits in selection

programs were generally limited (Rodenburg and Turner, 2012; Grandin and Deesing, 2014).

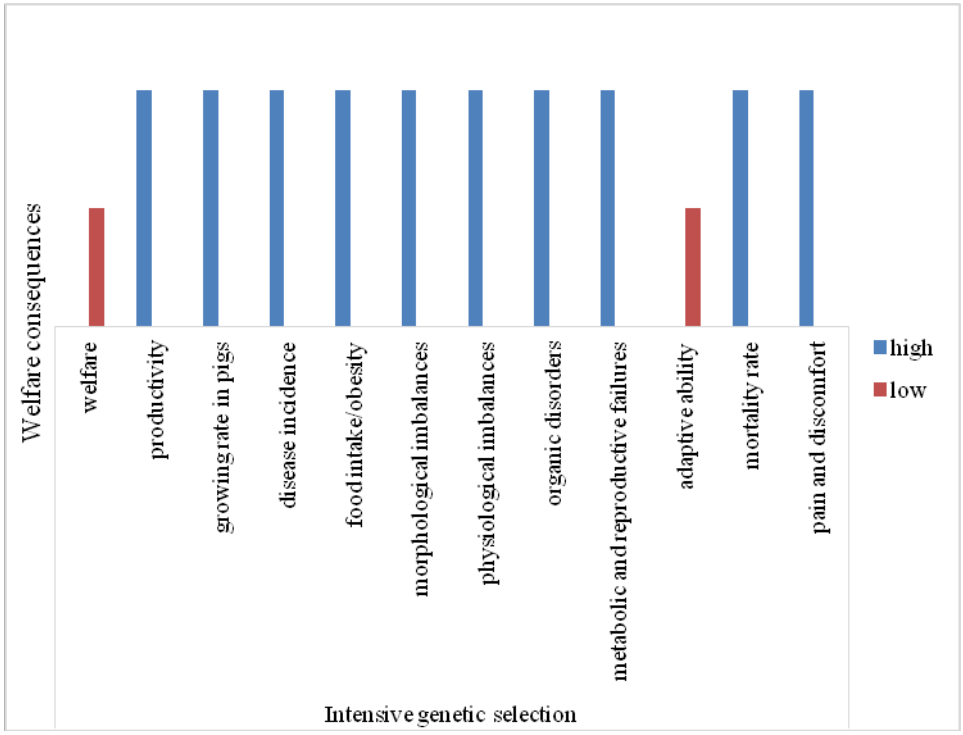

Figure 2. Welfare consequences regarding intensive genetic selection.

Genetic selection for high productivity is often associated with high food intake, which leads to the need for food restriction and high levels of starvation at certain stages of production. For example, pregnant sows (EFSA, 2007a) and parent broilers (de Jong et al., 2012) are no longer in the age when high rates of a weight gain are desired, so these animals are usually fed only about 50% *ad libitum* intake. If *ad libitum* is allowed to access the same concentrated feeds that are usually given, these animals will face an increased risk of metabolic and reproductive disorders (EFSA, 2009a, b). Therefore, the dilemma of choosing between hunger and obesity-related diseases clearly appears here, and both variants are unfavourable in terms of animal welfare (EFSA, 2007a; EFSA, 2009a, b; de Jong et al., 2012; Hötzel, 2014).

There are two other very important dilemmas of the animal welfare in pig production (Figure 3), related to the use of farrowing crates and tail docking. The first dilemma is related to the two main interrelated problems of pig welfare: the

death of the pig related to crushing and limiting the movement of sows in farrowing crates. These crates are implemented to reduce the crushing of the piglets but have a disadvantage of depriving of most normal behavioural forms and strategies, including turning around and building nests. Keeping pigs in free-range systems solves this problem, but often increases the mortality of the piglets due to mother's crushing. The piglets that spend more time in contact with sows are probably more exposed to crushing, but changes in freehold systems designed to encourage the piglets to spend more time out of the reach of sows (for example, by providing a warm, soft and non-slippery space for piglets) have not brought significant success. This problem is further complicated by the selection of larger litters, which has the effect of increasing the proportion of light piglets that are more prone to crushing (EFSA, 2007a).

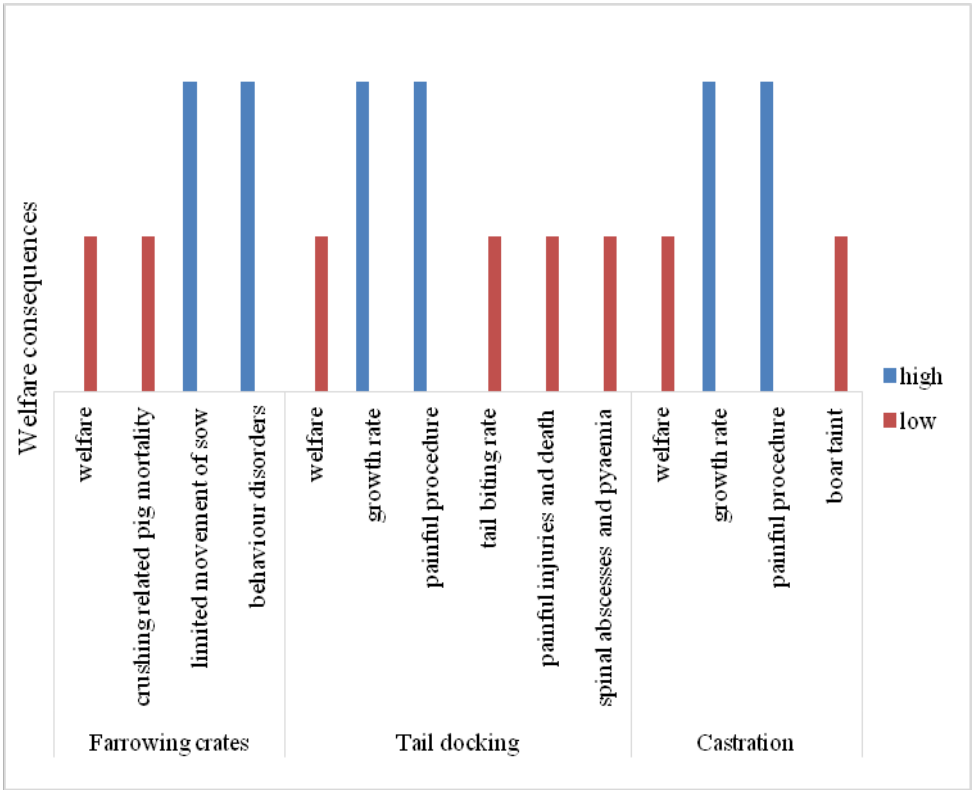

Figure 3. Welfare consequences in pigs.

The second well-known example of the animal welfare dilemma in pig breeding is the widespread practice of docking the tail in piglets in order to reduce

the risk of tail biting occurrence. Tail biting is common in confined systems and can cause painful injuries and deaths in growing piglets. Also, tail biting is accompanied by different pathological changes, varying from spinal abscesses to pyaemia in different body regions. These changes are often followed by a reduced growth rate or in more severe cases, total carcass condemnation (D'Eath et al., 2014, 2016; Valros et al., 2016; EFSA, 2007b, c). Although the tail docking is painful, anaesthesia is usually not applied. In addition to the immediate pain associated with this zootechnical procedure, at the point of amputation of the tail, neuromas and therefore chronic pain may occur frequently. Docking of the tail would not be necessary if the basic causes of tail biting were removed (EFSA, 2005a, 2007b, c). Risk factors associated with the occurrence of tail biting relate to large groups of piglets and high density of population, non-stimulating environment and genotype, even though the relative contribution of each of these factors has so far been little considered and explained in the research (EFSA, 2007a). Although European legislation (EC, 2001) requires the use of adequate stall space enrichment and the restriction of the use of tail docking with only exceptionally applicable cases, this zootechnical procedure is still common practice in many countries, which points out the difficulty of controlling this abnormal behaviour, especially in intensive rearing systems. For now, it is concluded that the docking of the tail has a tendency to reduce the appearance of tail bites and will be applied until this behaviour is explained and effective preventive measures are suggested; probably, it will continue to be practiced regardless of legal restrictions (Sonoda et al., 2013; Hötzel, 2014).

Castration in piglets, another major welfare problem, exists to this day, although many studies have been conducted. Namely, castration of male piglets is performed primarily in order to avoid the development of the unpleasant smell and taste of boar meat. Although Welfare Law says that castration could be performed without analgesia in the first seven days of life, it is painful at any age (EFSA, 2004).

In broiler production, one of the well-known dilemmas (Figure 4) is related to the paradox of parent broiler breeding: obviously, genetic progress in broiler growth efficiency will not slow until economics warrant slows improved efficiency and yield (de Jong, 2012; OIE, 2018e). EFSA (2010) scientific opinion explained the impact of genetic parameters which may affect the commercial broiler welfare. According to this, the major welfare issue based on genotype and influence of management factors may lead to inadequate welfare and occurrence of skeletal disorders, contact dermatitis, ascites and sudden death syndrome. Most of these are related to high growth rates of broilers. In addition, this points out that there are various interactions between the environment and the genotype, with a serious adverse effect on welfare regarding lighting regimes, litter management, dietary deficiencies and contamination, air quality and temperature. When the welfare risks

are assessed, the odds of a hazard and the level of the poor welfare effects and consequences of that exposure have to be estimated (EFSA, 2010; 2012a).

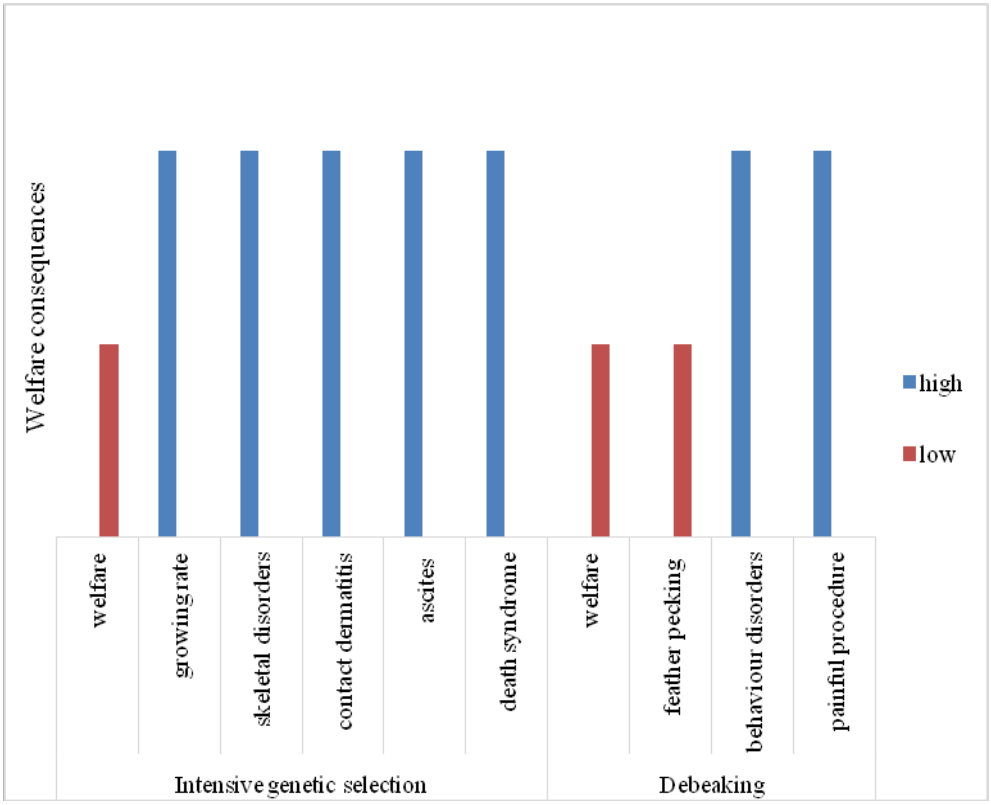

Figure 4. Welfare consequences in poultry.

Another important example of the dilemma regarding animal welfare is well-known debeaking in laying hens and the occurrence of feather pecking. In the prevention of feather pecking, debeaking is considered effective because the feather condition is deteriorated in laying hens with intact beaks, compared to the birds with trimmed beaks, although the feather pecking occurs also in debeaked flocks. It should be kept in mind that chickens with a shortened beak are less prone to ground peck and preening. The debeaking process itself is painful, and the creation of neuromas at the top of the shortened beak causes long-lasting pain. The cutting of the beak, therefore, does not solve the basic problem, but only deals with the consequences. Therefore, the breeding of chickens with intact beaks should be an integral part of sustainable laying hen production (Lambdon et al., 2010; Kaukonen and Valros, 2019).

On behalf of the European Commission, EFSA published scientific opinions on animal health and welfare of different categories of pigs in relation to housing and husbandry (EFSA, 2007a, b), risks accompanied with tail biting in pigs and suggested solutions to reduce the need for tail docking considering the different rearing systems (EFSA, 2007c), welfare aspects of the castration of piglets (EFSA, 2004) and effects of different floor types and space allowances on welfare of weaning and rearing pigs (EFSA, 2005a). The detailed scientific report on the effects of farming systems on dairy cow welfare and disease (EFSA, 2009a) was published, as well as the contemporary achievements regarding the risk assessment in respect to different solutions in housing, nutrition and feeding, management and genetic selection concerning dairy cow metabolic and reproductive problems (EFSA, 2009b). In the poultry production, the scientific report has been reviewed and updated on the welfare of broilers and broiler breeder (de Jong et al., 2012), as well as the scientific opinion on the welfare aspects of the use of perches for laying hens (EFSA, 2015c). The latest scientific knowledge on these topics was presented in all of these scientific opinions and reports, providing conclusions and recommendations in accordance with the previously defined requirements of the European Commission.

Botreau et al. (2007) presented the general criteria for animal welfare assessment. Having in mind the importance of animal-based indicators, since they define welfare from animal point of view, EFSA has considered their use in farm animals (EFSA, 2012c), pigs (EFSA, 2012d), dairy cows (EFSA, 2012d), broilers (EFSA, 2012e) and in small-scale farming systems (EFSA, 2015c). Besides this, an analysis of the gaps in the use of animal-based measures in the EU was realised (EFSA, 2015b). In addition, EFSA presented the outcome of the public attitude on the guidance on the risk assessment (EFSA, 2012f), guidance on animal welfare risk assessment (EFSA, 2012a) and scientific opinion on the welfare risks related to the sheep production (EFSA, 2014). In the above-cited publications, a crucial dilemma of farm animal welfare was considered, taking into account specific issues.

Many review papers are aimed to highlight the importance of the welfare friendly housing system planning, emphasising the necessity not only to explore the field of animal welfare, suggesting new and modified housing systems but to perform a survey of current but contentious systems as well, paying special attention to the design contributions to these systems in respect of farm animal welfare.

Also, areas for future research on ensuring farm animal welfare are highlighted in the literature. Von Keyserlingk et al. (2009) emphasised key concepts and the need to explore the welfare of dairy cattle, while Hemsworth (2018) presented key facts regarding management and housing design implications on pig welfare. According to von Keyserlingk et al. (2009), three major concerns of

animal welfare are: is the animal functioning well, is the animal feeling well, and is the animal able to live according to its nature? Issues in pig production include the following aspects: effectiveness of environmental enrichment for gestating sows in intensive, indoor and non-bedded systems, prospects to prolong foraging and feeding periods in feed-restricted gestating sows, propose accommodation options that allow both access to feed, water, comfortable lying area, and escape opportunities in order to reduce aggression, minimising risks to the welfare of group-housed sows, and less confined farrowing and lactation systems (Hemsworth, 2018). These papers point out that those animal welfare problems may be less a consequence of the type of housing system than of how well it operates. In addition, Lay et al. (2011) have concluded that the right combination of housing design, breed, rearing conditions, and management is essential to optimise hen welfare and productivity in different housing systems.

Dawkinis et al. (2004) analysed the influence of housing conditions and stocking density on chicken welfare. Their results show that differences among environment features for chickens that are provided by producers more affect welfare than stocking density itself. The skills, knowledge and motivation of stockmen to effectively care for and deal with their animals are essential for the welfare level. Stockmen attitude influences not only how they handle animals but also their motivation. Even though public concerns and policy debates are often focused on intensive housing systems, available data indicate that the design and management of both indoor and outdoor housing systems are probably more important for animal welfare than it is expected. Therefore, upgrading of technical skills and knowledge and the attitudes and behaviours of stockmen must be a primary goal of the human resource management practices at a farm (Vučinić, 2006; Hristov et al., 2007b; Broom and Fraser, 2007).

Regardless of the production system, it is necessary to provide minimum standards of welfare for all species and categories of farm animals. In this sense, the authors in our country described various aspects of animal welfare, such as farm animal welfare concept: from beginnings to integration in modern production systems (Ostojić-Andrić et al., 2018), the conditions of rearing, welfare and behaviour of farm animals (Hristov et al., 2006c), basic principles of dairy cattle welfare plan creation and implementation (Hristov et al., 2015a), welfare and behaviour in relation to disease of dairy cows (Hristov et al., 2015b), rearing conditions, health and welfare of dairy cows (Hristov et al., 2008), welfare of dairy cattle – current status and perspectives (Hristov et al., 2012b) and the welfare of dairy cattle on farms (Hristov and Stanković, 2016). All analysed papers basically encompass some aspects that clarify the dilemmas of the welfare of farm animals in intensive production.

In our country, in a number of papers, minimum standards of farm animal welfare were also reviewed, e.g. related to hygienic conditions for housing and

ensuring of poultry welfare in the European Union (Hristov, 2005), housing conditions and health care of goats (Hristov and Relić, 2005), conditions of housing and welfare of sheep and goats (Hristov et al., 2007c), housing conditions and welfare of cattle (Hristov et al., 2007e), housing conditions and welfare of laying hens (Pandurević et al., 2007), housing conditions and welfare of pigs (Petrović et al., 2007), welfare and biosecurity standards on farms focusing on housing conditions of cattle and pigs (Hristov et al., 2009) and hygienic standards in rearing of piglets (Hristov et al., 2006b). In general, stockmen in Serbia for years have had information about new technological trends in the production and farm animal welfare achievement, but they are often unable, for various reasons, to completely apply them. Because of this, farm animals often suffer from the deprivation of space, qualitatively and quantitatively inadequate nutrition, inability to express species-specific behaviour, infectious and non-infectious diseases that are frequent due to professional failures, as well as poor farm procedures, often implemented by less competent, unmotivated and usually underpaid employees. Housing conditions for animals are mainly determined by the financial possibilities of a particular breeder, who often uses inadequate materials such as concrete for the construction or renovation of buildings, without taking into account the needs of animals, their health, production results and the productivity life length (Vučinić et al., 2007; Hristov and Stanković, 2009a; Stanković et al., 2014; Ostojić Andrić et al., 2015; Ostojić Andrić et al., 2016b).

There is a need to identify the dilemmas of veterinarians during their surveys of the welfare incidents that involve stockmen facing numerous social, health and psychological problems. Three related dilemmas for veterinarians were revealed: defining professional parameters, determining the appropriate response and involvement versus detachment. It is a well-known fact that EU regulations on farm animal welfare are guided primarily by zoocentric approach and professional ability to recognise relevant animal-based indicators (Hristov et al., 2018). Study evidence shows that veterinarians are willing to assist the stockmen in order to ease animal suffering (Devitt et al., 2014).

Serbia: a state of the art

Up to now, several methods have been developed to assess the welfare of farm animals. Some of these methods became part of the legislation in many countries; to be more effective, they are being actively applied, supplemented and re-examined. That is the case with the following methods: Animal Needs Index (Bartussek et al., 2000), EFSA methods for the assessment of animal welfare risk (EFSA, 2012b, c, d, e f, EFSA, 2014, EFSA, 2015a) and protocols on the quality welfare assessment of cattle, pigs and laying hens (Welfare Quality®, 2009a, b, c). In the last 15 years, different aspects of methodology for assessing the welfare of

farm animals in our country have been considered: methodologies for assessing the welfare of dairy cows and pigs that have been developed within the project TR 20110, the most important indicators of dairy cow welfare evaluation (Hristov et al., 2012a), different approaches to assess the welfare of dairy cows with some results in Serbia (Hristov et al., 2014), assessment of conditions of housing and welfare of dairy cows (Maksimović et al., 2007), assessment of the welfare of cows in free housing (Hristov et al., 2011), welfare indicators of dairy cow focusing on selection and implementation in assessment (Ostojić Andrić et al., 2013), key health issues affecting dairy cow welfare (Ostojić Andrić et al., 2016a), behaviour of cattle as an indicator of their health and welfare (Relić et al., 2012), dairy cow health parameters in different seasons – a welfare approach (Ostojić Andrić et al., 2017), welfare and biosecurity indicator evaluation in dairy production (Hristov and Stanković, 2009b) and assessment of some welfare parameters in lactating sows (Relić et al., 2016). These papers provided very useful data for ensuring and improvement of farm animal welfare in Serbia.

In addition, the current problems related to the welfare of animals in Serbia (Vučinić et al., 2007), the most significant failures in ensuring the welfare of animals on farms of cattle and pigs (Hristov and Stanković, 2009c), the most common health disorders and welfare of dairy cows and calves (Stanković et al., 2014), welfare and behaviour in relation to disease of dairy cows (Hristov et al., 2015b), dairy cow welfare quality in loose vs. tie housing system (Ostojić-Andrić et al., 2011), housing conditions and welfare of dairy cows in Serbia (Ostojić Andrić et al., 2015), the state of welfare on Serbian dairy farms (Ostojić Andrić et al., 2016b), health and welfare of dairy cows in Serbia (Ostojić Andrić et al., 2016c), colostrum management in calves' welfare risk assessment (Relić et al., 2014), frequency of behavioural disorders of calves in the first month of life (Samolovac et al., 2018), influence of rearing conditions and birth season on calf welfare in the first month of life (Samolovac et al., 2019), as well as the appearance of feather loss in the laying hens as a welfare problem were considered (Hristov et al., 2006a). Although there are a number of problems directly related to the welfare of animals in Serbia, one of the basic is insufficient knowledge and skills of professionals and poor awareness of the citizens about this problem. The best solutions that can change the existing status of the welfare of animals and the awareness of citizens are training and education through the inclusion of all subjects competent to transfer knowledge and skills (Vučinić et al., 2007, Hristov and Stanković, 2009c; Stanković et al., 2014; Ostojić Andrić et al., 2015; Ostojić Andrić et al., 2016b).

Approaches to solving problems and dilemmas of the welfare of farm animals

In order to solve the farm animal welfare dilemmas, it is important to provide all the direct and indirect participants in the technological process of production

with professional ethical principles. Knowledge of the technological process of production, economic, legislative and scientific principles has great importance in improving the welfare of farm animals in all kinds of rearing systems. The easiest ways to improve stockmanship are to select employees carefully, and even better – to train them to improve their technical knowledge, working organisation and attitudes towards both animals and husbandry practices (Boivin et al., 2003; Hristov et al., 2007b).

To date, two approaches to addressing problems endangering farm animals welfare in all farming systems have been proposed (Vučinić, 2006). The first approach is based on manipulations in the living environment and its improvement (free system of rearing, increasing of space, enrichment of the environment), so animals can satisfy their basic behavioural needs. This method is based on the use of alternative housing systems that allow the expression of all forms of normal behaviour without altering the productivity of animals. The second approach is based on the use of behavioural principles of restraint, zootechnical procedures, veterinary interventions and the placement of farm animals in order to preserve well-being and prevent pain, distress and suffering (Dawkins et al., 2004; von Keyserlingk et al., 2009; Lay et al., 2011; Hemsworth, 2018). In addition, the application of genetic selection is required in scientific research in order to examine all aspects of animal adaptation to intensive rearing systems (Grandin and Deesing, 2014). Also, there are attempts to carefully apply some therapeutic procedures in order to influence the physical condition or psychological status of the animal and thus preserve their well-being (Vučinić, 2006).

It should be kept in mind that contradictions that accompany animal welfare include not only economic factors, but other factors as well. A multidisciplinary approach in animal welfare assurance context must be used, complementary to the food safety, environmental protection, worker health and safety, economics, international trade, domestic protection, public perception and consumer economics (McGlone, 2001; Anon., 2015; Anon., 2017; Anon., 2018). Demand for the production of more food in an environmentally sustainable way can affect the efficiency of production in relation to other goals, among which are the objectives of ensuring the welfare of animals. Prior to the higher scientist engagement in future animal welfare research, enabling larger quantities of cheaper food production in further sustainable system intensification, it should be considered whether this option corresponds to real improvements in the animal welfare and whether it is the best or at least a satisfactory option that can cope with current and future challenges in livestock production (Broom and Fraser, 2007).

Conclusion

Based on the considerations of the most important dilemmas regarding the ensuring of the welfare of farm animals, it could be concluded that the main dilemma concerns the question of whether extensive production is generally better compared to the intensive, and whether on the farms of a smaller capacity, better welfare of the animals in relation to larger farms is ensured. In addition, the next dilemma relates to the application of an intensive selection of farm animals and its impact on the emergence of problems in ensuring their welfare. The following dilemma is related to the two main interrelated problems of pig welfare and individual farrowing pens: the piglet death by crushing and the sows' movement restriction. One of the well-known welfare dilemmas is the paradox of parent flocks of broiler line breeding that could not be solved until the pressure for genetic advancement in production is required due to the economic efficiency; the next examples of animal welfare dilemmas are the widespread practice of tail docking in piglets in order to reduce the risk of tail biting and debeaking in the laying hens and the occurrence of feather pecking in free rearing systems. Finally, there are also dilemmas about the consumers' willingness to pay a higher price for the products that originate from animal welfare friendly conditions.

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NAJZNAČAJNIJE DILEME KOJE SE ODNOSE NA DOBROBIT FARMSKIH ŽIVOTINJA

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R e z i m e

U ovom preglednom radu razmatraju se kontradikcije u modernoj stočarskoj proizvodnji kao dileme koje se odnose na dobrobit farmskih životinja. Glavna dilema odnosi se na pitanje da li je ekstenzivna proizvodnja na malim farmama generalno bolja od intenzivne proizvodnje na velikim farmama. Sledeća dilema odnosi se na primenu intenzivne selekcije životinja i njen uticaj na nastanak problema dobrobiti. Naredna dilema se odnosi na dva glavna međusobno povezana problema dobrobiti svinja u pojedinačnim boksovima za prašenje: uginuća prasadi gnječenjem i ograničenje kretanja krmača. Takođe, dilema u vezi sa dobrobiti je paradoks roditeljskih jata tovnih pilića koja se ne može rešiti dok postoji pritisak za genetski napredak u proizvodnji zbog ekonomske efikasnosti. Sledeći primer dileme je široko rasprostranjena praksa sečenja repa kod prasadi kako bi se smanjio rizik od griže repova. Iako je sečenje repa bolno i može prouzrokovati uginuće, anestezija se obično ne primjenjuje na farmama. Sličan primer dileme je i skraćivanje kljuna kod kokoši nosilja i pojava kljucanja perja u sistemima slobodnog uzgoja. Važno je uočiti razliku između opisanih dilema, gde je jedna premisa suprotstavljena drugoj, što je u suštini konflikt između ciljeva dobrobiti životinja i drugih aspekata, kao što su ekonomska, etička i moralna pitanja. Konačno, postoje i neke dileme koje se odnose na spremnost potrošača da plate višu cenu za proizvode koji su dobijeni u uslovima gajenja koji pospešuju dobrobit farmskih životinja.

Ključne reči: dileme, zaštita dobrobiti, farmske životinje.

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PRODUKTIVNOST I KVALITET ZRNA SORTI TRITIKALEA PRI RAZLIČITIM KOLIČINAMA MINERALNE ISHRANE

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Sažetak: U radu je prikazan uticaj sorte i različitih doza primenjenog azota na prinos zrna i sadržaj proteina u zrnu ozimog tritikalea. Ogled je bio postavljen u periodu od 2010. do 2012. godine na severu Crne Gore, u okolini Bijelog Polja. Istraživanje je obuhvatalo 5 sorti ozimog tritikalea (Odisej, Kg-20, Trijumf, Rtanj i Tango) i sledeće varijante đubrenja: kontrola (bez đubrenja), samo azot u količini od 60 kg ha^{-1} i azot u količinama od 60 i 90 kg ha^{-1} u kombinaciji sa istom količinom fosfora i kalijuma (80 kg ha^{-1}). Najmanji prosečan prinos zrna dobijen je na varijanti bez đubrenja – kontroli. Upotreba đubriva, kod svih ispitivanih sorti, dovela je do vrlo značajnog povećanja prinosa na svim varijantama u poređenju sa kontrolom. Najmanji prosečan prinos ostvarila je sorta Kg-20, a najveći sorta Tango. Najveći sadržaj proteina kod svih sorti ostvaren je na varijanti đubrenja gde je upotrebljen samo azot u količini od 60 kg ha^{-1}. Najveći sadržaj proteina u zrnu, u proseku za sve varijante đubrenja, imala je sorta Trijumf. Podaci o ostvarenim prinosima i sadržaju proteina u zrnu, u zavisnosti od sorte i upotrebljene doze đubriva, govore o odlikama pojedinih sorti i mogu služiti kao kriterijum za izbor najpogodnije sorte za određene agroekološke uslove. Ovo je posebno značajno za gazdinstva orijentisana ka uzgoju stoke kojima obezbeđenje dovoljne količine kvalitetne hrane predstavlja prioritet.

Ključne reči: prinos, sadržaj proteina, genotip, azot, fosfor, kalijum.

Uvod

Tritikale (x Triticosecale Wittmack), stvoren ukrštanjem pšenice i raži, predstavlja hibridnu vrstu novog botaničkog roda u porodici *Poaceae*. Nastao sa idejom objedinjavanja pozitivnih osobina roditeljskih vrsta, tritikale poslednjih godina postaje sve zastupljeniji u proizvodnji. Mogućnost gajenja na većim

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nadmorskim visinama, na zemljištima lošijih fizičko-hemijskih osobina, zaslanjenim i kiselim zemljištima uz izraženu otpornost prema biotičkim i abiotičkim stresovima, samo su neke od osobina po kojima se tritikale izdvaja (Villegas et al., 2010; Frašet al., 2016). Takođe, zahvaljujući visokom sadržaju proteina, povoljnom aminokiselinskom sastavu i nutritivnim vrednostima većim i od kukuruza, tritikale predstavlja odličnu komponentu za spravljanje krmnih smeša za ishranu svih vrsta domaćih životinja (Đekić et al., 2009; 2014; 2019).

Za postizanje visokih prinosa zrna dobrog kvaliteta, neophodno je voditi računa o vremenu i gustini setve, unosu dovoljnih količina azota, fosfora i kalijuma i obezbeđenju maksimalne mikrobiološke aktivnosti u zemljištu. Prema navodima (Zečević et al., 2010; Bielski, 2015) đubrenje, a posebno ishrana azotom, jedna je od od najvažnijih agrotehničkih mera koja utiče na prinos zrna i obezbeđuje ostvarivanje visokog genetskog potencijala za prinos. Jaśkiewicz (2011) tvrdi da je jedino poznavanjem i ispunjavanjem agrotehničkih zahteva određene sorte moguće postići prinose koji su po visini bliski njenom maksimalnom genetskom potencijalu za prinos.

Upotreba azota u prihrani bez jasne dijagnostike i preciznog utvrđivanja količine može da dovede do suprotnih efekata od očekivanih. Nepotrebno povećanje lisne površine, uzrokovano viškom azota, povećava potrošnju vode, smanjuje otpornost biljaka prema biotičkim i abiotičkim stresovima, kao i otpornost prema poleganju, itd. (Bogdanović et al., 2005; Glamočlija et al., 2015). Weber et al. (2008), Pecio (2010) i Wang et al. (2017) ističu da na komponente prinosa osim doze upotrebljenog azota u velikoj meri utiče i vreme njegove primene. Prihrana biljaka azotom u pravo vreme direktno utiče i na visinu prinosa i na sadržaj proteina u zrnu (Kara i Uysal, 2009; Lestingi et al., 2010). Tritikale se najviše primenjuje kao stočna hrana. Brojna istraživanja ukazuju da tritikale uspešno zamenjuje deo kukuruza, pšenice ili ječma u stočnoj hrani bez negativnih posledica na prirast domaćih životinja, zahvaljujući visokom sadržaju proteina sa visokim sadržajem nezamenljivih aminokiselina, naročito lizina (Milovanović et al., 2007; Đekić et al., 2009; McGoverin et al., 2011).

Cilj ovog rada bio je da se utvrdi uticaj različitih doza primenjenog azota na prinos zrna i sadržaj proteina u zrnu sorti ozimog tritikalea.

Materijal i metode

Ogled je izveden u periodu od 2010. do 2012. u okolini Bijelog Polja (Crna Gora) na zemljištu tipa eutrični kambisol sa aluvijalnim nanosom. Istraživanjem je bilo obuhvaćeno pet sorti ozimog tritikalea poreklom iz različitih selekcionih kuća (sorta Odisej – stvoren u Institutu za ratarstvo i povrtarstvo u Novom Sadu, Kg-20 i Trijumf – sorte selekcionisane u Centru za strna žita u Kragujevcu i sorte Rtanj i Tango nastale u Centru za poljoprivredna i tehnološka istraživanja u Zaječaru). U

ogledu su bile zastupljene sledeće varijante đubrenja: kontrola (bez đubrenja) (0), samo azot u količini od 60 kg ha^{-1} i azot u količinama od 60 i 90 kg ha^{-1} u kombinaciji sa istom količinom fosfora i kalijuma (80 kg ha^{-1}). Ogled je bio postavljen po slučajnom blok sistemu u četiri ponavljanja, sa veličinom elementarne parcelice od 6 m^2 (3x2 m). Priprema zemljišta za setvu obuhvatala je osnovnu obradu u septembru i predsetvenu pripremu neposredno pre setve setvospremačem u drugoj polovini oktobra. Setva je u obe godine obavljena ručno u drugoj polovini oktobra, sa razmakom između redova od 12 cm. Upotrebljena su pojedinačna mineralna đubriva koja su uneta pre setve. Celokupna količina fosfora i kalijuma (80 kg ha^{-1}) i 1/3 predviđene količine azota uneti su pre setve, a ostatak azota do predviđene količine dat je u prihrani useva krajem marta. Žetva je obavljena ručno u fazi pune zrelosti. Posle žetve izmeren je prinos sa svake elementarne parcelice, preračunat na 14% vlage u zrnu i izražen u t ha^{-1}. Takođe, sa svake elementarne parcelice, uzeti su uzorci zrna za hemijsku analizu. Sadržaj proteina u zrnu određen je mikro metodom po Kjeldahlu u laboratoriji Centra za strna žita u Kragujevcu. Dobijeni rezultati o prinosu zrna i sadržaju proteina u zrnu ozimog tritikalea, obrađeni su analizom varijanse i izračunavanjem Pearson-ovog koeficijenta korelacije uz korišćenje statističkog paketa WASP 2.0.

Agroekološki uslovi

Zemljište

Zemljište predstavlja izuzetno dinamičnu sredinu, jedan je od najvažnijih elemenata biljne proizvodnje i osnovni je preduslov visokih i stabilnih prinosa (Biberdžić et al., 2018; Adesola et al., 2018). Zemljište na kome je izveden ogled pripada tipu eutrični kambisol na aluvijalnom nanosu. Većina ovih zemljišta u dolini Lima sadrže male količine $CaCO_3$, a mogu biti i beskarbonatna usled čega reakcija zemljišta može varirati u širokom rasponu. Usled sporije mineralizacije humusa u uslovima hladnije klime, ovo zemljište je dosta humozno (3,35–3,96%), sa niskim sadržajem fosfora i kalijuma. Prema pH vrednosti, zemljište je kisele reakcije (tabela 1).

Tabela 1. Hemijske osobine zemljišta.
Table 1. Chemical properties of the soil.

Dubina (cm)/ *Depth (cm)*	pH		$CaCO_3$ (%)	Humus (%)	P_2O_5 (mg na 100 g^{-1} zemlj.)	K_2O (mg na 100 g^{-1} zemlj.)
	H_2O	nKCl				
0–10	5,61	5,01	2,4	3,35	5,12	7,5
10–30	5,53	4,94	2,44	3,96	4,24	3,8

Vremenski uslovi u toku izvođenja ogleda

Klima za šire područje opštine Bijelo Polje može se okarakterisati kao kontinentalna sa velikim uticajem okolnih visokih planina. Karakteristike klime ovog područja su visoka relativna vlažnost vazduha tokom cele godine, hladne zime sa ekstremno niskim temperaturama u toku decembra i januara i pojava kasnih prolećnih i ranih jesenjih mrazeva kao i olujnog vetra praćenog gradom, što predstavlja ograničavajući faktor za gajenje velikog broja poljoprivrednih kultura.

U tabeli 2 prikazane su količine padavina po mesecima i srednje mesečne temperature vazduha u toku vegetacionog perioda tritikalea. Količina padavina u toku prve vegetacione sezone bila je za 213 mm veća u poređenju sa drugom vegetacionom sezonom. U periodu od oktobra 2010. godine do jula 2011. godine, najviše padavina je bilo u decembru, a najmanje u martu, dok je u drugoj godini najveća količina padavina zabeležena u februaru, a najmanja u novembru. Takođe, tokom poslednja tri meseca u godini koja su od značaja za setvu i početni razvoj biljaka, u prvoj godini je bilo 343 mm padavina, dok je u istom periodu u drugoj godini istraživanja količina padavina iznosila svega 98 mm. Nedostatak padavina u novembru 2011. godine, kao i obilne snežne padavine u februaru 2012. godine, ispoljile su negativan uticaj prvo na početni razvoj biljaka, a onda su usporile vegetaciju u proleće, što je za posledicu imalo kasnije klasanje i cvetanje biljaka i pad prinosa.

Tabela 2. Mesečne količine padavina (mm) i srednje mesečne temperature vazduha (°C) za područje opštine Bijelo Polje.
Table 2. Monthly precipitation (mm) and average monthly air temperatures (°C) for the municipality of Bijelo Polje.

Godina/*Year*	Meseci/*Months*										Prosek/*Average*
	X	XI	XII	I	II	III	IV	V	VI	VII	
Količina padavina (mm)/*Precipitation*											
2010–11	65	131	147	36	76	31	46	121	33	79	765
2011–12	36	7	55	79	183	57	47	46	34	8	552
1961–90	80	115	91	87	68	60	70	76	72	64	783
Srednja mesečna temperatura vazduha (°C)/*Average monthly air temperature*											
2010–11	10,12	8,54	2,05	-0,65	0,94	6,03	10,54	14,5	18,9	21,23	9,2
2011–12	9,3	3,25	2,17	-1,72	-3,52	5,96	10,8	15,02	20,67	24,63	8,7
1961–90	9,4	4,7	0,2	-1,3	0,7	4,9	9,0	13,3	16,3	17,9	7,5

Za razliku od prve godine kada je srednja mesečna temperatura vazduha u novembru iznosila 8,54°C, niske temperature u istom mesecu naredne godine usporile su klijanje, nicanje i početni porast biljaka. Razlike u temperaturi uočene su i u februaru, pa je srednja mesečna temperatura u 2011. godini iznosila 0,94°C, a

u 2012. godini -3,52°C. Povoljne temperature u maju i junu praćene zadovoljavajućom količinom padavina u 2011. godini omogućile su pravilno nalivanje zrna. Znatno veće temperature vazduha tokom juna i jula 2012. godine uz skromne rezerve vlage iz prethodnih meseci, dovele su do skraćenja perioda nalivanja zrna i ubrzanog zrenja, što je za posledicu imalo manji prinos u poređenju sa prethodnom godinom istraživanja.

Rezultati i diskusija

Razlike u visini prinosa zrna bile su uslovljene ne samo sortama, već i količinom upotrebljenih đubriva kao i godinama istraživanja. U proseku, za sve ispitivane sorte u obe godine istraživanja, najveći prinos zrna postignut je na varijanti gde je azot upotrebljen u količini od 90 kg ha^{-1}, ali je isti bio statistički značajno veći u odnosu na kontrolnu i N_1 varijantu đubrenja. Najniži prinosi zrna kod svih sorti zastupljenih u ogledu zabeleženi su na varijanti bez đubrenja.

Sve sorte su brojčano najveći prinos zrna postigle na varijanti koja je podrazumevala upotrebu najveće količine azota sa izuzetkom sorte Tango u prvoj i sorte Kg-20 u drugoj godini istraživanja koje su najveće prinose ostvarile na N_2 varijanti đubrenja. Međutim, te razlike nisu bile statistički značajne (tabela 3).

Tabela 3. Analiza varijanse prinosa zrna sorti tritikalea na različitim varijantama đubrenja.
Table 3. Variance analysis of grain yield of triticale varieties in different fertilizer variants.

ANOVA Table									
Izvor varijac./*Source of var.*	Ss/*Df*	Suma kvad./*Sum of squ.*	Sr. kvad./*Mean sq.*	F izr./*F cal.*	F ver./*F prob.*	Suma kvad/*Sum of squ.*	Sr. kvad./*Mean sq.*	F izr./*F cal.*	F ver./*F prob.*
Replik./*Repl.*	2	0,163	0,081	0,946	0,397	0,031	0,015	0,267	0,767
Tret./*Treatme.*	19	45,370	2,388	27,764	0,000	34,228	1,801	31,402	0,000
Sorte/*Var. (A)*	4	13,067	3,267*	37,983**	0,000	2,913	0,728	12,694**	0,000
Đubr./*Fert. (B)*	3	31,065	10,355	120,401*	0,000	30,907	10.302	179,586**	0,000
A x B	12	1,238	0,103	1,199**	0,319	0,408	0,034	0,592**	0,834
Greška/*Error*	38	3,268	0,086	-	-	2,180	0,057	-	-
Ukupno/*Total*	59	-	-	-	-	-		-	-

Izvor varijacije/*Source of variation*; Replikacija/*Replication*; Tretmani/*Treatments*; Sorte/*Varieties*; Đubrenje/*Fertilization*; Ss – Stepeni slobode/Df – *Degrees of freedom*; Suma kvadrata/*Sum of squares*; Sredina kvadrata/*Mean squares*; F izračunato/*F calculated*; F verovatnoća/*F probability.*

Do sličnih rezultata su došli i Wojtkowiak et al. (2013), ističući da je prinos zrna varirao u zavisnosti od doze primenjenog azota, ali i da zabeležene razlike nisu bile statistički značajne. Značajan efekat primene kompletnog hraniva (NPK), u odnosu na primenu samo azota, rezultat je niže pH vrednosti ovog tipa zemljišta,

kao i niskog sadržaja lako pristupačnog fosfora i kalijuma koji se iz tih razloga moraju dodavati u obliku đubriva. Fosfor i kalijum iz upotrebljenih đubriva se sporije kreću kroz zemljište, nisu podložni ispiranju i najčešće ostaju u onom sloju gde su i uneti. Potrebne količine ovih elemenata se određuju na osnovu hemijske analize u zemljištu i njihovog balansa u biljkama (Jelić i Kostić, 1994; Glamočlija et al., 2015).

Takođe, zapaženo je i da su značajan uticaj na visinu prinosa ispoljili i meteorološki uslovi u godinama istraživanja, te je u skladu sa tim prosečan prinos za sve sorte i primenjene varijante đubrenja bio veći u prvoj u poređenju sa drugom godinom ispitivanja.

Za razliku od prve godine, znatno manja količina padavina u oktobru i novembru 2011/12. godine uslovila je kasnije i nepotpuno nicanje, a obilne snežne padavine u februaru i dugo zadržavanje snežnog pokrivača iste godine usporile su vegetaciju u proleće, pa su faze klasanja i cvetanja nastupile kasnije. Uz napred pomenuto, veće temperature vazduha u junu i julu 2012. godine uzrokovale su skraćenje perioda nalivanja zrna te ubrzano zrenje i smanjenje prinosa. Da visina prinosa zrna zavisi od uslova godine, genotipa i primenjene agrotehnike, konstatovali su ranije i Jelić et al. (2015), Terzić et al. (2018) i Đekić et al. (2019).

Tabela 4. Srednje vrednosti prinosa zrna sorti tritikalea na različitim varijantama đubrenja.
Table 4. Mean values of grain yield of triticale varieties in different fertilization variants.

Prinos zrna (t ha^{-1})/*Grain yield (t ha^{-1})*										
(A) Sorte/*Varieties*	2010/2011					2011/2012				
	(B) Varijanta đubrenja/*Fertilization variant*									
	0	N_1	N_2	N_3	$\bar{X}$	0	N_1	N_2	N_3	$\bar{X}$
Odyssey	3,75	4,30	5,01	5,08	4,53	3,12	4,03	4,99	5,04	4,29
Kg-20	3,12	3,99	4,36	4,70	4,04	2,99	3,54	4,52	4,39	3,86
Trijumf	3,66	4,58	5,63	5,69	4,89	3,23	4,08	4,80	5,00	4,28
Rtanj	3,63	4,86	5,55	5,68	4,93	3,34	4,11	4,99	5,11	4,39
Tango	3,96	5,15	6,27	6,20	5,39	3,40	4,13	5,16	5,30	4,50
$\bar{X}$	3,62	4,58	5,36	5,47	4,76	3,22	3,98	4,89	4,97	4,26
LSD	A		B	AxB		A		B	AxB	
0,05	0,242		0,217	0,485		0,198		0,177	0,396	
0,01	0,325		0,290	0,649		0,265		0,237	0,530	

0 – kontrola; N_1 – 60 kg ha^{-1} N; N_2 – 60 kg ha^{-1} N, 80 kg ha^{-1} P_2O_5, 80 kg ha^{-1} K_2O; N_3 – 90 kg ha^{-1} N, 80 kg ha^{-1}P_2O_5, 80 kg ha^{-1} K_2O.

Značajan uticaj na visinu prinosa ispoljili su i meteorološki uslovi u godinama istraživanja, te je u skladu sa tim prosečan prinos za sve sorte i primenjene varijante đubrenja bio veći u prvoj u poređenju sa drugom godinom ispitivanja.

Za razliku od prve godine, znatno manja količina padavina u oktobru i novembru 2011/2012. godine uslovila je kasnije i nepotpuno nicanje, a obilne snežne padavine u februaru i dugo zadržavanje snežnog pokrivača iste godine usporile su vegetaciju u proleće tako da su faze klasanja i cvetanja nastupile kasnije. Pored toga, više temperature vazduha u junu i julu 2012. godine uzrokovale su skraćenje perioda nalivanja zrna te ubrzano zrenje i smanjenje prinosa. Na osnovu svojih istraživanja, Jelić et al. (2015), Biberdžić et al. (2017), Terzić et. (2018) i Đekić et al. (2019) zaključili su da visina prinosa zrna zavisi od uslova godine, genotipa i primenjene agrotehnike.

Jedna od najvažnijih pozitivnih osobina tritikalea jeste visok sadržaj proteina. Rezultati ovih istraživanja pokazali su da je sadržaj proteina u zrnu najviše zavisio od sorte, zatim od vremenskih uslova u godinama ispitivanja kao i od varijante đubrenja (tabele 5 i 6). U obe godine ispitivanja, sorta Trijumf je imala veći prosečan sadržaj proteina u zrnu u poređenju sa ostalim ispitivanim sortama tritikalea. Najmanji prosečan sadržaj proteina u zrnu u prvoj godini istraživanja zabeležen je kod sorti Odisej i Rtanj (11,99 %), dok je u drugoj godini najmanji sadržaj proteina u zrnu imala sorta Tango (13,48 %). Milovanović (1997), Bruckner et al. (1998) i Đekić et al. (2011) ističu da su u njihovim istraživanjima najmanji sadržaj proteina imale najprinosnije sorte tritikalea kao i da su na sadržaj proteina veliki uticaj imali klimatski uslovi u godinama ispitivanja.

Tabela 5. Analiza varijanse sadržaja proteina u zrnu (%) na različitim varijantama đubrenja.
Table 5. *Variance analysis of grain protein content (%) in different fertilizer variants.*

ANOVA Table									
Izvor varijac./*Source of var.*	Ss/*Df*	Suma kvad./*Sum of squ.*	Sr. kvad./*Mean sq.*	F izr./*F cal.*	F ver./*F prob.*	Suma kvad/*Sum of squ.*	Sr. kvad./*Mean sq.*	F izr./*F cal.*	F ver./*F prob.*
Replik./*Repl.*	2	0,261	0,131	22,591	0,000	0,040	0,020	1,690	0,198
Tret./*Treatme.*	19	72,661	3,822	660,902	0,000	94,051	4,950	417,346	0,000
Sorte/*Var. (A)*	4	12,768	3,192	551,946**	0,000	13,831	3,458	291,538*	0,000
Đubr./*Fert. (B)*	3	52,830	17,610	3045,019**	0,000	75,665	25,222	2126,472**	0,000
A x B	12	7,023	0,585	101,191**	0,319	4,555	0,380	32,000**	0,834
Greška/*Error*	38	0,220	0,006	-	-	0,451	0,012	-	-
Ukupno/*Total*	59	-	-	-	-	-		-	-

Izvor varijacije/*Source of variation;* Replikacija/*Replication;* Tretmani/*Treatments;* Sorte/*Varieties;* Đubrenje/*Fertilization;* Ss – Stepeni slobode/*Df – Degrees of freedom;* Suma kvadrata/*Sum of squares;* Sredina kvadrata/*Mean squares;* F izračunato/*F calculated;* F verovatnoća/*F probability.*

Prosečan sadržaj proteina u prvoj godini bio za 1,50% manji u odnosu na isti u drugoj godini koja je sa aspekta količine padavina i temperatura bila nepovoljnija (tabela 6). Prema Milovanoviću (1997), sadržaj proteina u zrnu u velikoj meri

zavisi od vremenskih uslova u kritičnim fazama rasta i razvića. Autor takođe navodi da smežuranost zrna koja nastaje kao posledica vodnog stresa praćenog visokim temperaturama u pomenutim fazama dovodi do narušavanja odnosa između endosperma koji je bogat skrobom i omotača bogatijeg proteinima, stoga se takva zrna karakterišu većim sadržajem proteina (Fernandes-Figares et al., 2000).

Tabela 6. Sadržaj proteina u zrnu (%) na različitim varijantama đubrenja.
Table 6. Grain protein content (%) in different fertilizers variants.

Sadržaj proteina (%)/*Protein content (%)*										
Sorte (A)/*Varieties*	2010/2011					2011/2012				
	Varijanta đubrenja (B)/*Fertilization variant (B)*									
	0	N_1	N_2	N_3	$\overline{X}$	0	N_1	N_2	N_3	$\overline{X}$
Odyssey	10,00	12,69	12,55	12,72	11,99	12,06	15,37	14,37	14,56	14,09
Kg-20	11,87	14,37	12,87	12,60	12,93	11,94	14,87	14,25	14,17	13,81
Trijumf	12,06	14,44	13,00	12,91	13,10	12,61	16,49	15,25	15,06	14,85
Rtanj	10,06	13,19	12,35	12,37	11,99	11,87	14,50	14,12	14,03	13,63
Tango	11,00	13,31	12,69	12,34	12,33	11,94	13,75	14,12	14,10	13,48
$\overline{X}$	10,99	13,60	12,69	12,59	12,47	12,08	14,99	14,42	14,38	13,97
LSD	A		B	AxB		A		B	AxB	
0,05	0,063		0,056	0,126		0,090		0,080	0,180	
0,01	0,084		0,075	0,168		0,121		0,108	0,241	

0 – kontrola; N_1 – 60 kg ha^{-1} N; N_2 – 60 kg ha^{-1} N, 80 kg ha^{-1} P_2O_5, 80 kg ha^{-1} K_2O; N_3 – 90 kg ha^{-1} N, 80 kg ha^{-1} P_2O_5, 80 kg ha^{-1} K_2O.

Primena đubriva dovela je do značajnog povećanja sadržaja proteina u zrnu kod svih ispitivanih sorti u poređenju sa kontrolom, pri čemu je najveći sadržaj proteina (14,29%) zabeležen na varijanti sa najmanjom količinom azota (14,29%). Do sličnih rezultata su došli i Sekeroglu i Yilmaz (2001), koji ističu da je najveći sadržaj proteina u zrnu tritikalea dobijen pri upotrebi 80 kg ha^{-1} azota, te da dalje povećanje količine azota nije pozitivno uticalo na ovo svojstvo. Na varijanti gde je primenjena najmanja količina azota ostvaren je značajno manji prinos u odnosu na ostale varijante. U ovim istraživanjima, povećanje prinosa dovelo je do smanjenja koncentracije azota u zrnu i smanjenje sadržaja proteina, što su ranije u svojim istraživanjima utvrdili i Bruckner et al. (1998) i Alaru et al. (2003). Sadržaj azota u zrnu zavisi i od međusobnog odnosa hraniva u primenjenim đubrivima. U skladu sa tim, upotreba azota u kombinaciji sa kalijumom i NPK đubriva sa odnosom hraniva 1:1:1 povećava sadržaj proteina u zrnu, dok povećan udeo drugih hraniva u odnosu na N snižava sadržaj proteina (Tyrone, 2002; Jelić et al., 2004), što su potvrdila i ova istraživanja.

Između visine prinosa i sadržaja proteina u zrnu postojala je srednje jaka negativna korelacija (r=-0,65). Prema istraživanjima Milovanovića (1992; 1997),

Brucknera et al. (1998) i Alaru et al. (2003), najmanji sadržaj proteina su imale najprinosnije sorte tritikalea, što potvrđuju i rezultati ovih istraživanja.

Zaključak

U obe analizirane godine, sorte tritikalea su se značajno razlikovale u prinosu zrna. Sve sorte su ostvarile veći prinos u prvoj godini zbog povoljnijih vremenskih uslova. U proseku za godine, najveći prinos ostvarila je sorta Tango, a najmanji sorta Kg-20. Primena đubriva dovela je do značajnog povećanja prinosa kod svih sorti u odnosu na kontrolu. U proseku za sve sorte, najveći prinos zrna dobijen je primenom 90 kg ha^{-1} azota u kombinaciji sa istom količinom fosfora i kalijuma (80 kg ha^{-1}) i isti je bio značajno veći u odnosu na kontrolu i varijantu gde je upotrebljen samo azot u količini od 60 kg ha^{-1}. Najveći sadržaj proteina u zrnu ustanovljen je kod sorte Trijumf u obe godine istraživanja. Između visine prinosa i sadržaja proteina utvrđena je negativna korelacija.

Rezultati ovih ispitivanja ukazuju na to da svaka sorta zahteva određene uslove klime, zemljišta i ishrane. Kako bi se maksimalno ispoljio potencijal za prinos zrna, s obzirom na to da tritikale dobija sve značajnije mesto u ratarskoj proizvodnji u poljoprivrednim uslovima severa Crne Gore, kao vredna komponenta pri spremanju koncentrovane hrane za ishranu domaćih životinja, sorte Tango i Trijumf mogu se preporučiti za širu proizvodnju.

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PRODUCTIVITY AND QUALITY OF GRAINS OF TRITIKALE VARIETIES AT VARIOUS QUANTITIES OF MINERAL NUTRITION

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A b s t r a c t

This paper presents the influence of varieties and different doses of applied nitrogen on grain yield and protein content of triticale. The experiment was set in the period from 2010 to 2012 in the north of Montenegro, in the vicinity of Bijelo Polje. The research included 5 varieties of winter triticale (Odyssey, Kg-20, Triumph, Rtanj and Tango) originating from different breeding houses and the following varieties of fertilization: control (without fertilization), only nitrogen in the amount of 60 kg ha-1 and nitrogen in the amount of 60 and 90 kg ha-1 in combination with the same amount of phosphorus and potassium (80 kg ha-1). The results of the study showed that the lowest average grain yield was obtained in the non-fertilizing variant – control. The use of fertilizers in all tested varieties has led to a very significant increase in yield in all variants compared to control. The Kg-20 variety had the lowest average yield, and the Tango variety had the highest. The highest average protein content was achieved in the fertilizer variant where only nitrogen was used in the amount of 60 kg ha-1. Among the researched varieties, the Triumph variety had the highest protein content in the grains. The data on the achieved yields and the content of protein in grains, depending on the variety and the used doses of fertilizer, indicate the characteristics of individual varieties and can serve as a criterion for the selection of the most suitable variety for certain agroecological conditions. This is particularly important for cattle-oriented farms, where the main priority is to ensure a sufficient amount of quality food.

Key words: grain yield, protein content, genotype, nitrogen, phosphorus, potassium.

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GROWTH AND YIELD RESPONSE OF OKRA (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH) TO FERTILIZER TYPES AND TIMES OF APPLICATION IN THE SOUTHERN GUINEA SAVANNA AGRO-ECOZONE OF NIGERIA

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Abstract: A field study was carried out at the Kwara State University Teaching and Research Farm, Malete, to evaluate the response of vegetative growth and immature fruit yield of okra (*Abelmoschus esculentus* (L.) Moench) to three fertilizer types and three times of application. The fertilizer types, used at the rate of 60 kgN/ha, were Kwasu organic-based fertilizer type 2 (KOBF-2), Aleshinloye organo-mineral fertilizer grade A (AOMF-A), and inorganic NPK 15-15-15 fertilizer (NPK), each applied at planting time, 2 weeks after planting (WAP), and 4 WAP. The trial was laid out in a 3 x 3 factorial arrangement in a randomized complete block design and replicated three times. All the data collected were subjected to analysis of variance, using the SAS statistical package, and the treatment means were separated using Duncan's Multiple Range Test at 5% probability level. The result indicated no significant differences in plant height, number of leaves/plant, number of fruits/plant, and the average length of the fruits between fertilizer types and application times. However, there were significant differences in leaf area/plant at 6WAP and fruit yield. Irrespective of the times of application, KOBF-2 gave significantly higher immature fruit yield (2.84 t/ha) than NPK (1.93 t/ha) and AOMF-A (1.72 t/ha). The results thus suggest that the new organic-based fertilizer formulated in this study (KOBF) deserves incorporation into national fertilizer programs for sustainable crop production.

Key words: okra, vegetative growth, fruit yield, inorganic fertilizer, organic-based fertilizer.

Introduction

Okra is a popular fruit vegetable in tropical and sub-tropical countries of the world, including Nigeria where it is cultivated majorly for its immature fruit, which is consumed either in the fresh or dried form (Dupriez and De Leener, 1989; NIHORT, 2000; Schippers, 2000). The tender okra fruit is mostly used to prepare a

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mucilaginous or 'draw' soup, which is relished in Nigeria because it greatly eases swallowing of staple starchy morsel foods prepared from yam, cassava roots, and the cereals (NIHORT, 2000). The culinary and nutritive values of the immature okra fruit are due to its rich store of physiologically valuable nutrients, especially water (88.0%), soluble carbohydrates (9.5%, mainly as dietary fiber in the form of mucilage), protein (2.1%), and fair amounts of several minerals (zinc, calcium), vitamins (A, B2, B6, ascorbic acid, folic acid, riboflavin, thiamin), and antioxidants (Fatokun and Chedda, 1983; Grubben and Denton, 2004).

Ordinarily, there are two cultivated okra genotypes in Nigeria: the daylength-sensitive West African okra (*Abelmoschus caillei* (A. Chev.) Stevels) and the common okra (*Abelmoschus esculentus* (L.) Moench). Regardless of the time of planting, during the rainy season, the former does not start to initiate flowering and fruiting until October and, therefore, it is usually cultivated at subsistence level towards the end of the rainy season. The common okra, in turn, is the more commonly cultivated and it has received far more genetic improvement and agronomic studies; the reasons why several improved varieties of it are widely available for cultivation in market gardens and mixed cropping systems (Kochhar, 1986; NIHORT, 2000; Schippers, 2000).

In addition to the use of high-quality seeds of improved varieties for planting the crop, high level of soil fertility and crop protection are other prerequisites for high yield of qualitative okra fruits. NIHORT (2000) has posited that the quantum of okra fruit yield depends to a large extent on the ability of the farmer to replenish plant nutrients removed from the soil by the current and previous crops, maintaining the physical condition of the soil at desirable levels, and preventing undue increases in soil acidity and toxic mineral elements. Maintenance of soil fertility, therefore, is a major consideration in okra production, which in conventional agriculture, is routinely met by the use of inorganic fertilizers.

It is not far-fetched why most farmers prefer inorganic to organic fertilizers. Inorganic fertilizers are readily available, convenient to handle especially during the application, and contain a higher concentration of plant nutrients per unit weight. Notwithstanding these advantages, it is common knowledge that use of inorganic fertilizers, in the long run, poses serious soil environmental pollution and ecosystem balance problems, in addition to systematic impoverishment of the soil and degradation of soil physical properties, all of which lead to crop yield decline (Avery, 1995; Palm and Sanchez, 1991). Also, inorganic fertilizers are becoming increasingly unavailable to resource-poor farmers as a result of ever-rising cost and transportation constraints from urban centers, where they are mainly obtainable, to where they are required in the rural areas.

These setbacks of inorganic fertilizers have given rise to the current advocacy for the adoption of organic and organic-based fertilizers for soil fertility maintenance in crop production systems. These fertilizer types are not only safe for

the health of farmers, consumers, and the soil environment but usually leave positive residual effects on the soil for the growth and yield of succeeding crops, which benefits the farmer by way of reduced cost of fertilizing a succeeding crop (Worthington, 2001; Bayu et al., 2006; Makinde and Ayoola, 2008).

Olowoake and Adeoye (2013) have observed that several types of plant residues in Nigeria can be processed, packaged, and made available as organic-based fertilizers. Among such organic fertilizers in Nigeria, some of which have been evaluated on crop growth and yield, only a few are developed in the country exemplified by Aleshinloye organic fertilizer grades A and B (products of Aleshinloye Fertilizer Plant, Ibadan, Oyo State, Nigeria) and Sunshine organic fertilizer grades A and B (developed by Ondo State Government, Akure, Nigeria). While Aleshinloye grade A and Sunshine grade A are composted household wastes amended with mineral fertilizers, Aleshinloye grade B and Sunshine grade B are unamended composts (ODSG, 2012).

Prompted by the need to expand the range of locally-formulated organic-based fertilizers in Nigeria, crop scientists at the Kwara State University Malete (Kwasu) decided to experiment on mixing tithonia plant (*Tithonia diversifolia* L.), poultry manure, and inorganic NPK fertilizer together. The resultant product is called Kwasu organic-based fertilizer (KOBF). The choice of tithonia, a roadside weed having vigorous growth habit, as a component in formulating KOBF was based on its availability virtually everywhere and its potential for raising the levels of the major nutrients in soils (Achieng et al., 2010; Jama et al., 2000). This attempt of the Kwara State University Malete (Kwasu) crop scientists was also informed by the general agreement among several researchers that using a mixture of organic and inorganic fertilizers for soil fertility management significantly performs better on growth and yield of crops than using the single fertilizer types alone (Makinde et al., 2001; Sridhar and Adeoye, 2003; Makinde and Ayoola, 2008; Ogunlade et al., 2011).

Earlier studies on organic and organic-based fertilizers (Akanbi et al., 2000; Olowoake and Adeoye, 2013; Olowoake, 2014) have shown that their potentials are not limited only to sustaining soil fertility, but also extend to greatly improving the physical properties of tropical soils and activating and increasing soil microbial populations that carry out decomposition of soil organic matter, leading to improved crop growth and yields. However, there is no such information on KOBF, because it is a relatively new organic fertilizer. The objective of this study, therefore, is to evaluate the response of okra growth and fruit yield to time of application of KOBF compared to some of the commonly available organic-based and inorganic fertilizer types in Nigeria.

Materials and Methods

Study location

The study was conducted at the Kwara State University Teaching and Research Farm, Malete, Nigeria. Malete is located in the southern Guinea savannah agro-ecological zone of Nigeria, on latitude $08^{O}71'$N and longitude $4^{O}44'$E of the equator. The local climate is characterized by distinct wet and dry seasons. The wet season commences in March or April and ends in October, with a dry spell from mid-July to mid-August. The dry season starts towards the end of October and lasts until March or April. Meteorological data of the study location during 2017 are presented in Table 1.

Analysis of study location soil and organic-based fertilizers used in the study

Samples of the study location soil were collected at a depth of 0–30 cm, before the commencement of the trial, for laboratory analysis of physico-chemical properties. KOBF-2 and AOMF-A were also analyzed in the laboratory for their nutrient compositions.

Planting material

The okra seeds used for planting were those of Clemson spineless variety; produced, treated, and hermetically packaged by Premier Seed Company Ltd., with germination percent of 99.

Experimental design and layout

Three fertilizer types and three times of application were tested in this study. The fertilizer types were Kwasu organic-based fertilizer type 2 (KOBF-2), Aleshinloye organo-mineral fertilizer grade A (AOMF-A), and inorganic NPK 15-15-15 fertilizer (NPK), each applied at three planting times: 0 WAP, 2 WAP, and 4 WAP. Thus, the trial was laid out in a 3 x 3 factorial structure in a randomized complete block design and replicated three times. Each plot was 3.0m x 3.0m in size with 1.0m alleys between the plots and the replicates, respectively.

Cultural practices

The experimental site was plowed, harrowed, and the plots and replicates pegged out. Three seeds were hill-planted at a spacing of 50cm between and within rows. Seedlings took 4–5 days to begin emerging and were thinned to two healthy

and vigorous plants per stand at 2 WAP. Weighed quantities of each of the three fertilizer types, equivalent to 60kgN/ha (Sonkuwar et al., 1997), were applied to designated plots and plant stands by placement and burial about 5 cm from the hole or plant base at 0, 2, and 4 WAP. To forestall activity of leaf-eating and pod-boring insect pests, dichloro-divenyl-phosphate (DDVP) insecticide was applied as a foliar spray at the rate of 50ml/50 liters of water, beginning from 2 WAP and at fortnight interval until harvest commences. Weed control in the plots was performed by regular hoeing and hand pulling.

Data collection and analysis

Using non-destructive methods, the following data, expressed as the mean per plant, were taken from each plot on 4 randomly selected plants from among the 4 plant stands in the innermost rows of each plot: plant height taken with a meter rule from ground level up to the tip of the topmost leaf buds on the main stem; number of expanded leaves per plant; area of the leaves per plant, estimated as the mean of the product of measured length and breadth of each of 4 leaves per plant, then multiplied by a coefficient given by Ross (1967) as 0.69.

Data on immature fruit yield and yield components were collected by harvesting with a sharp knife at a 4-day interval for five consecutive times between 51 and 71 days after planting. The following data were taken: number of fruits per plot, mean individual fruit length, and total fruit yield per plot and treatment, extrapolated to kg/ha. All weight determinations were carried out on a portable top-loading laboratory weighing scale model OHAUS-Traveller. The data collected were subjected to analysis of variance, using the SAS statistical package and the treatment means, where significant, were separated using Duncan's Multiple Range Test at 5% probability level.

Results and Discussion

Climatic elements during the period of the study

Rainfall, temperature and relative humidity observed during the period of the experiment were adequate for okra cultivation. Statistics obtained from the hydrology section of Lower Niger River Basin Development Authority, Ilorin (a few kilometers from the study location) show that in 2017 (Table 1) rain commenced in March and lasted until October with a total of 123.03 cm delivered. Relative humidity during the rainy season ranged between 22.4% and 25.1%, and temperature between 25 and 30^{O}C.

Analysis of soil and organic-based fertilizers

Table 2 shows the physical and chemical properties of the study location soil and the nutrient compositions of the organic-based fertilizers used in this study. The soil was sandy loam and slightly acidic with a pH value of 6.29 in the water. The values of total nitrogen (1.82 mg/kg), available phosphorus (8.48 mg/kg), and potassium (0.88 mg/kg) in the soil were below the critical values for soils of the Guinea savanna (Olowoake and Ojo, 2014). The low content of the major nutrients indicated in the results signifies the low soil organic matter content and the need for nutrient supplementation in order to achieve optimum plant performance (Azeez et al., 2006). Analysis of the organic-based fertilizers indicated that KOBF-2 had substantially more N, P, and K than AOMF-A, in addition to some other micro-nutrients.

Table 1. Meteorological data of the study location during 2017.

Months	Rainfall (cm)	Temperature (°C)	Relative humidity (%)
January	2.20	27.06	20.96
February	0.00	28.54	21.78
March	33.50	30.60	25.01
April	12.73	29.15	25.08
May	8.44	27.62	24.65
June	12.32	27.03	23.83
July	4.30	29.09	23.57
August	18.74	25.01	22.65
September	20.27	25.66	22.41
October	12.73	26.85	23.24
November	0.00	28.05	23.14
December	14.00	27.79	21.75

Source: Lower Niger River Basin Development Authority, Ilorin (Hydrology Section, 2017).

Vegetative growth of okra as affected by fertilizer type and application time

The responses of the vegetative growth characters of okra to the three fertilizer types and times of application are presented in Tables 3 and 4. Each of the vegetative growth characters measured (plant height, number of leaves and leaf area per plant) increased numerically in magnitude as growth progressed, but the response was not significant between the fertilizer types except for leaf area per plant at 6WAP, which was higher for KOBF-2 than NPK and AOMF-A. Considering the application time of each of the fertilizer types, the vegetative characters of okra were not significantly influenced ($p \leq 0.05$) by the three times of application. However, in numerical terms, 2 WAP gave higher values than when

the fertilizer was applied at planting (0 WAP) and 4 WAP for leaf area per plant using KOBF-2 and NPK but not AOMF-A.

Table 2. Compositions of the study location soil and organic-based fertilizers.

	Study location soil	Kobf-2	Aomf-a
Physical properties			
Sand (%)	58.69		
Silt (%)	19.31		
Textural class	Sandy loam		
Chemical properties			
pH (H_2O)	6.29	6.48	
Organic carbon (g kg^{-1})	1.54	3.59	
Available P (mg kg^{-1})	8.48	5.55	0.8
Total N (mg kg^{-1})	1.82	4.41	1.2
Exchangeable bases			
K (mg kg^{-1})	0.88	3.21	2.9
Na (mg kg^{-1})	1.98	1.28	
Mg (mg kg^{-1})	1.82	1.21	
Mn (mg kg^{-1})	1.02		
Zn (mg kg^{-1})	2.87		
Cu (mg kg^{-1})	0.87		
Ca (mg kg^{-1})	2.46	8.48	
Fe (mg kg^{-1})	3.01		

KOBF-2 = Kwasu organic-based fertilizer type 2; AOMF-A = Aleshinloye organo-mineral fertilizer grade A.

Table 3. Effects of the application time of different fertilizers on vegetative growth characters of okra.

Treatments		Plant height (cm)			No. of leaves/plant			Leaf area/plant (cm^2)		
Fertilizer type	Application time	2WAP	4WAP	6WAP	2WAP	4WAP	6WAP	2WAP	4WAP	6WAP
KOBF-2	0 WAP	6.53a	11.03a	25.03a	3.17a	4.93a	7.00a	5.63a	56.10a	230.53ab
	2 WAP	5.50a	9.07a	23.40a	3.00a	4.90a	7.27a	2.17a	54.43a	269.83a
	4 WAP	6.37a	10.20a	22.67a	3.10a	5.70a	7.27a	3.77a	47.77a	195.23ab
AOMF-A	0 WAP	5.43a	12.90a	13.43b	2.77a	5.37a	5.93a	1.77a	32.37a	128.57b
	2 WAP	5.83a	9.33a	18.87ab	3.00a	5.07a	5.83a	2.86a	30.30a	140.07b
	4 WAP	6.17a	9.77a	20.47ab	3.17a	5.43a	5.77a	3.63a	42.93a	169.77ab
NPK	0 WAP	5.93a	9.43a	19.93ab	3.00a	5.37a	6.10a	3.10a	39.93a	137.97b
	2 WAP	5.70a	11.07a	23.10a	3.10a	5.20a	6.37a	4.47a	46.13a	187.97ab
	4 WAP	6.40a	10.10a	20.93ab	3.10a	5.07a	6.53a	5.86a	37.33a	162.90ab

Means in the same column followed by at least one common letter are not significantly different. KOBF-2 = Kwasu organic-based fertilizer type 2; AOMF-A = Aleshinloye organo-mineral fertilizer grade A; NPK = Inorganic NPK 15-15-15 fertilizer; WAP = Weeks after planting.

Irrespective of the time of application (Table 4), KOBF-2 gave significantly higher values at 6 WAP than AOMF-A and NPK for all the vegetative characters measured, except the number of leaves per plant where values recorded for KOBF-2 were similar with NPK. At this time also, irrespective of fertilizer type, application at planting time (0 WAP) gave numerically but not significantly lower values than application at 2 and 4 WAP for plant height and leaf area per plant in AOMF-A and NPK.

Table 4. Effects of fertilizer type and time of application on vegetative growth characters of okra.

Treatments	Plant height (cm)			No. of leaves/plant			Leaf area/plant (cm^2)		
Fertilizer type	2WAP	4WAP	6WAP	2WAP	4WAP	6WAP	2WAP	4WAP	6WAP
KOBF-2	6.13	10.10	23.70a	3.09	5.12	7.12a	3.87	52.77a	231.87a
AOMF-A	6.01	10.20	17.59c	2.98	5.29	5.84b	2.77	35.20c	146.13b
NPK	5.81	10.67	21.32bc	3.01	5.21	6.33ab	4.48	41.13ab	162.94b
Application time									
0WAP	5.97	11.12	19.47	2.98	5.22	6.34	3.50	42.80	165.69
2WAP	5.68	9.82	21.79	3.03	5.06	6.49	3.16	43.62	199.29
4WAP	6.31	10.02	21.37	3.12	5.40	6.52	4.42	42.68	175.97
S.E.	NS	NS	1.52	NS	NS	0.31	NS	5.23	19.15

Means in the same column followed by at least one common letter are not significantly different. KOBF-2 = Kwasu organic-based fertilizer type 2; AOMF-A = Aleshinloye organo-mineral fertilizer grade A; NPK = Inorganic NPK 15-15-15 fertilizer; WAP = Weeks after planting; S.E. = Standard error.

Fruit yield and yield components of okra as affected by fertilizer types and application time

The effects of application time of the fertilizer types tested on immature fruit yield and yield components of okra are presented in Tables 5 and 6. There were no significant ($p \leq 0.05$) differences in the number of fruits per plot and the average length of the fruits among the fertilizer types and times of application. Although more fruits with longer length were recorded with the application of KOBF-2, compared to AOMF-A and NPK (Table 5), the values were not significantly different. However, fruit yield was significantly influenced by fertilizer type at various times of application. For instance, there was no significant difference between the three application times of KOBF-2 but AOMF-A at planting was superior to 2 and 4 WAP whereas, for NPK, application at 2 WAP was found to be superior at planting. Overall, the highest fruit yield (3.42 t/ha) was obtained with KOBF-2 applied at 2WAP and the least (1.38 t/ha) with NPK at 0 WAP.

Among the fertilizer types tested, irrespective of the time of application (Table 6), KOBF-2 gave significantly higher fruit yield (2.84 t/ha) than NPK and AOMF-

A, both having 1.93 and 1.72 t/ha, respectively. With respect to the time of fertilizer application, 2 WAP was found to be superior to 0 and 4 WAP.

Table 5. Effects of application time of different fertilizers on immature fruit yield and yield components of okra.

Fertilizer type	Application time	No. of fruits	Fruit length (cm)	Immature fruit yield (t/ha)
KOBF-2	0WAP	15.67a	9.03a	2.20ab
	2WAP	14.67a	7.30a	3.42a
	4WAP	16.67a	8.83a	2.90ab
AOMF-A	0WAP	10.00a	7.03a	2.17b
	2WAP	10.66a	7.23a	1.54c
	4WAP	10.67a	7.57a	1.46c
NPK	0WAP	12.00a	7.76a	1.38c
	2WAP	15.33a	7.80a	2.57b
	4WAP	13.33a	7.10a	1.83bc

Means in the same column followed by at least one common letter are not significantly different. KOBF-2 = Kwasu organic-based fertilizer type 2; AOMF-A = Aleshinloye organo-mineral fertilizer grade A; NPK = Inorganic NPK 15-15-15 fertilizer; WAP = Weeks after planting.

Table 6. Effects of fertilizer type and time of application on immature fruit yield and yield components of okra.

Treatments	No. of fruits	Fruit length (cm)	Immature fruit yield (t/ha)
Fertilizer type			
KOBF-2	15.67	8.39	2.84a
AOMF-A	10.44	7.56	1.72c
NPK	13.55	7.28	1.93bc
Application time			
0 WAP	12.57	7.94	1.92bc
2 WAP	13.56	7.92	2.51a
4 WAP	13.56	9.83	2.06bc

Means in the same column followed by at least one common letter are not significantly different. KOBF-2 = Kwasu organic-based fertilizer type 2; AOMF-A = Aleshinloye organo-mineral fertilizer grade A; NPK = Inorganic NPK 15-15-15 fertilizer; WAP = Weeks after planting; S. E. = Standard error.

A general overview of the results in this study shows that plant height and number of leaves as well as the area of the leaves per plant, except leaf area per plant at 6WAP, of the okra variety used in this study (Clemson spineless) were not significantly different between the fertilizers tested. The fruit yield components measured (number of fruits per unit area of land and mean individual fruit length) also showed a similar response as the above-mentioned vegetative growth

characters. The non-significant difference in the number of leaves is probably because the character is orthogenic in nature and, therefore, not readily subject to environmental influence.

The superiority of KOBF-2 applied at 2WAP over NPK and AOMF-A also applied at 2 WAP on leaf area per plant at 6WAP may be attributable to the relative differences in ease of mineralization of the component materials in these fertilizer types. KOBF-2, being a mixture of the tithonia plant and poultry manure amended with little inorganic fertilizer, proved to release its nutrients more readily, raising the levels of the major nutrients in the soil. This has agreed with Makinde et al. (2001) and Akanbi et al. (2000) that mixing organic and inorganic fertilizers is a sound soil fertility management strategy, better than the use of the organic and inorganic fertilizers individually. NPK is entirely an inorganic fertilizer, characterized by a rapid release of its constituent nutrients in the soil. AOMF-A, in turn, being from majorly household refuse, tends to mineralize more slowly compared to the other fertilizer types tested. In other similar studies, Sridhar and Adeoye (2003) and Ogunlade et al. (2011) have reported that the application of combined plant materials, poultry manure, and inorganic fertilizers significantly improves growth and fruit yield of okra better than the application of each fertilizer material separately.

Although the vegetative characters of okra were not significantly influenced by the three fertilizer application times tested, in numerical terms, values for leaf area per plant at 2 WAP (199.29 m^2) were higher than at planting time (165.69 m^2) and at 4 WAP (175.97 m^2). This may be attributed to the fact that when applied at planting time, the levels of available nutrients from each of the fertilizers must have declined drastically as growth progressed such that they were no more sufficient when needed most to sustain growth and yield. The higher overall values observed between the fertilizers when applied at 2 WAP suggest that mineralized nutrients were still available in sufficient amounts in the soil to support optimum okra plant growth and yield of fruits. However, at 4 WAP application time, probably the fertilizers have not yet mineralized sufficiently to make enough nutrients available at the time of flowering and fruiting. Abdulmaliq et al. (2015) have observed a similar result.

Application of AOMF-A at planting time gave significantly higher fruit yield values (2.17 t/ha) than at 2 and 4 WAP (1.54 and 1.46 t/ha, respectively), suggesting that this organo-mineral fertilizer requires a relatively long period of time after application to mineralize and make its constituent nutrients fully available for plant use. Namely, application times of 2 and 4 WAP appear not long enough for its complete mineralization and nutrient release for the use of the crop. AOMF-A is derived from majorly urban household refuse (ODSG, 2012), which, as shown in this study, tends to mineralize more slowly compared to the other fertilizer types tested.

For KOBF-2, irrespective of the time of application, immature fruit yields among the three fertilizer application times were not significantly different, although, in numerical terms, application at planting time gave lower fruit yield (2.20 t/ha) than at 2 and 4 WAP (3.42 and 2.90 t/ha, respectively), suggesting that this organic-based fertilizer readily mineralized and made its constituent nutrients available in the soil, with 2 WAP appearing to be more promising.

The effects of application times of NPK, in turn, closely followed those of KOBF-2. Application of NPK fertilizer to the okra crop at 2 WAP gave significantly higher fruit yield (2.57 t/ha) than at planting time (1.38 t/ha), although numerically lower it was not significantly different from when the fertilizer was applied at 4 WAP (1.83 t/ha). NPK as inorganic fertilizer is characterized by rapid mineralization and release of its constituent nutrients in the soil for plant uptake. Moyin-Jesu (2007) has observed that, compared to organic fertilizers, inorganic fertilizers quickly release their nutrients to the soil.

Therefore, the lower okra fruit yields obtained in this trial when NPK was applied at planting time, compared to at 2 WAP, suggest that available nutrients in the soil have depleted as a result of the long period of time (7 weeks) between the placement of the fertilizer and the commencement of fruiting. Similarly, the lower fruit yields when NPK was applied at 4 WAP, compared to at 2 WAP, suggest suboptimal levels of nutrients in the soil due to the short period of time (2 weeks) before fruiting commenced at 7 weeks, meaning that not enough of the fertilizer has been mineralized and the nutrients made available to the okra plants. These results are in agreement with the findings of Idowu and Kadiri (2013) and Iyagba et al. (2013) that the fresh fruit yield parameters of an okra crop (number of fruits/plant, fruit length, fruit weight/plant, and fruit weight/ha) are linked to the availability of adequate amounts of nutrients in the soil, especially N, P, and K, as at the time of flowering and fruiting.

Conclusion

Overall, the performances of the 3 fertilizer types tested can be ranked in the order KOBF-2 > NPK15-15-15 > AOMF-A. However, with respect to the time of application, KOBF-2 applied at 2 WAP was found to perform better than NPK, which in turn was superior to AOMF-A. The new organic-based fertilizer formulated in this study (KOBF) is thus recommended for incorporation into national fertilizer programs for sustainable crop production.

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RAST I PRINOS BAMIJE (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH) U ZAVISNOSTI OD TIPA ĐUBRIVA I VREMENA PRIMENE U AGRO-EKO ZONI JUŽNOGVINEJSKE SAVANE U NIGERIJI

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R e z i m e

Sproveden je poljski ogled na Nastavno-istraživačkom dobru Državnog univerziteta u Kvari, Malete, kako bi se procenio odgovor vegetativnog rasta i prinosa nezrelog ploda bamije (*Abelmoschus esculentus* (L.) Moench) na tri tipa đubriva i tri vremena primene. Tipovi đubriva, u količini od 60 kgN/ha, bili su Kwasu organsko đubrivo tipa 2 (KOBF-2), Aleshinloye organsko-mineralno đubrivo stepena A (AOMF-A), i neorgansko NPK 15-15-15 đubrivo (NPK). Sva navedena đubriva su primenjena u vreme setve, 2 nedelje posle setve (engl. *weeks after planting* – WAP), i 4 nedelje posle setve. Ogled je postavljen u 3 x 3 faktorijalnom rasporedu u potpunom slučajnom blok dizajnu u tri ponavljanja. Svi podaci koji su prikupljeni obrađeni su analizom varijanse, korišćenjem SAS statističkog paketa, a srednje vrednosti tretmana odvojene su korišćenjem Dankanovog testa višestrukog raspona na nivou od 5% verovatnoće. Rezultat je pokazao da nema značajnih razlika u pogledu visine biljke, broja listova po biljci, broja plodova po biljci, i prosečne dužine plodova između tipova đubriva i vremena primene. Međutim, postojale su značajne razlike kod površine lista po biljci 6 nedelja posle setve i prinosa ploda. Bez obzira na vreme primene, upotrebom KOBF-2 postignut je značajno viši prinos nezrelog ploda (2,84 t/ha) nego upotrebom NPK (1,93 t/ha) i AOMF-A (1,72 t/ha). Rezultati dakle sugerišu da novo organsko đubrivo formulisano u ovom istraživanju (KOBF) zaslužuje da bude uključeno u nacionalne programe o đubrivima za održivu proizvodnju useva.

Ključne reči: bamija, vegetativni rast, prinos ploda, neorgansko đubrivo, organsko đubrivo.

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MORPHOLOGICAL AND SSR MARKER CHARACTERIZATION OF WILD AND CULTIVATED COWPEAS (*VIGNA UNGUICULATA* L. WALP)

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Abstract: Three hundred and ninety accessions comprising 260 cultivated and 130 wild cowpea accessions were evaluated phenotypically using 27 cowpea descriptors. Morphological evaluation of some qualitative traits revealed 11.92% and 29.23% presence of pigmentation on the stem, 1.53% and 20.76% presence of stripes on the pod, and 0% and 20% presence of hairiness on the plant of cultivated and wild cowpeas respectively. As for the molecular analysis, sixteen SSR primers were employed for genotyping 48 accessions from both wild and cultivated cowpeas. The data generated a dendrogram with three clusters, two of which consisted of wild cowpea while the third cluster comprised all the cultivated cowpeas, including the yard-long-bean (*Vigna unguiculata* subsp. *sesquipedalis)* and *Vigna unguiculata* subsp. *cylindrica* accessions. Two wild accessions of subsp. *dekindtiana,* and one each of subsp. *kgalagadensis* and *protracta* clustered with cultivated cowpea indicating their relationships with cultivated cowpea, but not with other wild cowpeas. The numbers of polymorphic SSR bands in cultivated and wild cowpeas were 38 and 54, respectively, while the PIC values were 4.47 and 6.14, respectively, showing a greater genetic diversity in wild than in cultivated cowpeas. The subsp. *dekindtiana* had the highest number (80%) of shared SSR bands with cultivated cowpea followed by subsp. *protracta* with 54% of shared bands. Five species of wild cowpea have hairs and so could be used in breeding for resistance to insects.

Key words: cowpea, characterization, dendrogram, genetic diversity, microsatellites.

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Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most ancient human food sources and has probably been used as a crop plant since Neolithic times. Its origin has been linked to Africa based on the extent of diversity existing among germplasm lines and the preponderance of wild relatives distributed in several parts of the continent (Xiong et al., 2016). However, it has been found distributed, adapted and grown in many countries which include many countries in tropical Africa, Asia and South America (Mahalakshmi et al., 2007).

Annually, in the developing worlds, cowpea feeds millions of peoples, provides income for smallholder farmers, is used as animal fodder, helps restore soil fertility and gives comparatively high yields in harsh environments (Amusa et al., 2019). Annual estimated world production of cowpea worldwide is about 7.64 million tonnes, 60% of which is produced in Nigeria (FAO, 2017). Despite this, several authors have raised a concern about production instability over the years as compared to what is obtainable under experimental conditions (Singh, 2005; Kamara et al., 2012; FAO, 2017; Amusa et al., 2019).

Germplasm collection is important for the maintenance of biological diversity and food security. Germplasm can range from a collection of wild species to elite, domesticated breeding lines that have undergone extensive human selection. Thousands of accessions have been collected by crop genebanks or germplasm collection stores. The conservation and characterization of these genetic resources are a necessity not only for posterity but also for utilization in different improvement programs which include yield improvement, development of pest and disease tolerant and resistant genotypes, to mention a few (Kandel and Shrestha, 2018).

DNA markers can assist in producing a more robust and acceptable characterization of this economic crop by assessing the level of genetic relationships. In particular, microsatellite (SSR) markers, which are known to have a high level of variation among taxa and with a variable number of copies that are tandemly repeated, could be a useful tool in assessing the relationships between cultivated and wild cowpea. Microsatellites are inherited as codominant and are generally dispersed throughout the genome of organisms. Microsatellites have been used extensively in studying diversity at both intra- and interspecies levels of several crops such as alfalfa (ref), eggplant (Nunome et al., 2003), wild cowpea (Ogunkanmi et al., 2008), vegetable cowpea (Ogunkanmi et al., 2007), grapevine (Fan et al., 2014), tomato (Cheng et al., 2016; Arguirre et al., 2017) and *Brassica* species (Thakur et al., 2018), providing a mechanism for populations to adapt to their ever-changing environment. Hence, the objective of the study was to characterize the wild and cultivated accessions of cowpea to assess the level of diversity within Nigerian cowpea accessions.

Materials and Methods

Three hundred and ninety accessions within the section catiang of the subgenus *Vigna* were collected from the genebank of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. This included three subspecies of cultivated cowpea and 15 wild subspecies of cowpea covering a wide geographical range of Africa and part of Asia (Table S1). The cultivated members of this selection comprised one cylindrical and seven *sesquipedalis* accessions from Asia, and 260 accessions of subspecies *unguiculata* from Africa. The remaining 130 accessions of wild cowpea were from a wide geographical range in Africa, representing countries from southwest, northeast, central, and southern Africa. An augmented complete randomized block design was used for the experimental field layout. This consisted of 15 rows, 30 m by 2 m in dimension. There were thirty accessions per row, while each accession was replicated five times with two plants per stand 50 cm apart. The 50-cm spacing was designed to give enough space to each stand and enable them to fully establish on the field without excessive interference. Among these 30 accessions, there were four diverse cultivated cowpea checks (TVu 14176, TVu 14869, Tvx 3236, and TVu 9151) planted as per the recommendations of IITA's cowpea breeding program.

Morphological characterization was carried out using cowpea descriptors described by Padulosi et al. (1993) with slight modifications. A total of twenty-seven quantitative and qualitative characters were evaluated and analyzed using SAS software v 9.0 (2002).

Twenty-four accessions each from cultivated and wild cowpeas were selected for DNA analysis (Table S2). The selection was based on the distribution of accessions in the dendrogram constructed from the morphological data. At least one accession per cluster was selected from the dendrogram. Four seeds of each accession were sown in pots containing good loamy soil in a screen house at IITA, Ibadan, Nigeria. Two weeks after planting, newly opened fresh young leaves were picked per accession for DNA extraction. Extra leaves from the same plant were collected in polyethylene bags and stored at -80^{o}C in the freezer as a backup. Each leaf sample was placed in a 1.5 ml Eppendorf tube, quickly frozen in liquid nitrogen, and ground with Konte pestles into a fine powder. DNA was extracted from the ground leaves of each selected accession (N = 48) according to the procedure described by Dellaporta et al. (1983). The DNA was diluted in 0.1 × TE (1mM Tris 0.1mM EDTA, pH 8.0) to 10 ng/µl concentration.

Seventy SSR primers designed for cowpea by Li et al. (2001) were screened and optimized for polymorphism and annealing temperature using two accessions each from cultivated and wild cowpeas to ensure optimal primer performance across accessions. Optimal PCR amplification across accessions was achieved with annealing temperature ranging between 54 °C and 64 °C. Primers that showed

many bands, monomorphic bands or did not amplify were not selected for use in this study. Sixteen primers that showed unambiguous and clear polymorphism with the PCR products were used for this study.

A 10 µl reaction volume containing 1.0 µl of 10 × buffer, 2.0 µl of 10 ng/µl template DNA, 1.0 µl $MgCl_2$, 0.8 µl mixture of 10mM dNTPs (dATP, dCTP, dGTP, and dTTP), 4.6 µl of ultra-pure water, 0.5 µl each of forward and reverse primers, and 0.1 µl red hot *Taq* (promega) was loaded in a Perkin Elmer MJ cycler for DNA amplification. The PCR reaction was carried out with a profile of 18 cycles at 94°C for 1 min of initial denaturing and extension at 72°C for 1 min. Annealing temperatures were progressively decreased by 0.5°C every cycle from 64°C to 54°C. The reactions continued for 30 additional cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and ended with a 10-min extension at 72°C after about 3 hours. Microsatellite markers that showed polymorphism after screening on a 3% agarose gel were resolved for better resolution using polyacrylamide gel electrophoresis.

PCR products were separated on polyacrylamide gel containing 70 ml of freshly prepared 6% polyacrylamide solution, 350 ml of ammonium persulphite (APS), and 35ml of TEMED. The gel was run at a constant voltage of 65 volts and the current of 60 mA for 3 hours. The gel was later fixed, stained, and developed using a silver staining kit (Promega corp. Madison WI). Fragments that were resolved on gels (Figure 1) were scored as 1 or 0, i.e., the presence or absence of bands, respectively, on all the forty-eight accessions of cultivated and wild cowpeas. The bands that could not be confidently scored were regarded as missing data.

Pairwise distance (similarity) matrices were computed using sequential, hierarchical, and nested (SAHN) clustering option of the NTSYS-pc version 2.02j software package (Rohlf, 1993). The program generated a dendrogram, which grouped the test lines on the basis of Nei genetic distance (Nei, 1972) using unweighted pair group by using mathematical average (UPGMA) cluster analysis (Sneath and Sokal, 1973). Genetic distances were calculated from the SSR data as:

$$GD_{xy} = 1- (2N_{xy}/N_x+N_y), \quad (1)$$

where N_x and N_y are the numbers of alleles in groups X and Y, respectively, and N_{xy} is the number of alleles shared between the two groups of cultivated and wild cowpeas (Nei and Li, 1979). The correspondence between the two distance matrices (wild and cultivated cowpea lines) was assessed using simple linear correlation, cluster analysis, and principal component analysis. The polymorphism information content (PIC) provides an estimate of the discriminatory power of locus or loci by taking into account not only the number of alleles that are expressed but also the relative frequencies of those alleles. PIC values were calculated by the algorithm: PIC = 1-SnPi^2 where i starts from 1, Pi^2 = frequency

of the i^{th} allele (Brown et al., 1996; Ott, 1999). Only the distributions of the accessions along the first two principal components were considered in this paper.

Results and Discussion

In the study, twenty-seven characters were used to examine the relationships between the wild and cultivated cowpeas. It was observed that some members of the wild cowpea were found to share most of characters of cultivated cowpea and these included *dekindtiana* (TVNu 979, 305, 988, 578, 359, 1819, 315, 314, 582 669, 525, 245, 436, and 712) and *protracta* accessions (TVNu 288, 284, 390, and 267). This is in agreement with what was obtained by DNA analysis where they always aligned with the cultivated cowpea group.

For the vegetative characters, petiole length (plt) was highly correlated with rachis length (rlt) and petiolule. Results from the evaluation of some qualitative characters are presented in Table 1. The results revealed that there are more accessions (38) with purple pigmentation on their stems in wild cowpea than in cultivated cowpea (31). As for the presence of stripes on pods, cultivated cowpea had a higher percentage than wild cowpea. Accessions with the prostrate type of plant habit were more prominent than the number of accessions of erect and sub-erect type in both wild and cultivated cowpeas. Hairiness is one of the traits possessed by wild cowpea and this may correlate with their ability to resist insect pest attack. However, none of the cultivated cowpeas in this study had the trait, and hence may be the probable reason for their susceptibility to an insect pest. All the *vexillata* (14) used in this study and one accession of *rhomboidea* (TVNu 1471) showed the presence of hairs on stem and leaves. Meanwhile, some accessions of wild cowpea also possessed hairs but only on stems, and these included 5 *dekindtiana* (TVNu 305, 389, 255, 438, and 1589), two *protracta* (TVNu 505 and 273), one *trilobata* (TVNu 953), one *grandiflora* (TVNu 539), and the only two *pubescens* (TVNu 538 and 533) accessions used in this study.

Evaluation of some qualitative characters revealed that the proportion of pigmentation in wild cowpea was greater than in cultivated cowpea where the majority of the accessions had no pigment on stems. The ratio of pigment on the stem to non-pigmented stem in wild cowpea was approximately 1:3 while it was about 1:7 in cultivated cowpea (Table 1). A total of 37 accessions had an average of 20–30 leaves per plant, a large leaf area, and a copiously branched plant when examined at four weeks after planting. Thirty-four of them were cultivated cowpeas while the rest were wild cowpeas, which are all *dekindtiana* (TVNu 710, 249, and 303) accessions. This is a particular trait of agronomic importance as any of these accessions could be used as a forage crop for livestock animals. Possession of hairs in some accessions identified in this work could be a useful agronomic trait for insect-resistant improvement in cowpea. Pubescence, which is prominent in

some wild species, has been reported in several crop species and confers superior agronomic or abiotic stress adaptation. Agwaranze (1992) has observed that soybeans with dense pubescence are better adapted to high radiation, high temperature, and limited moisture conditions than those with less pubescence. He has stressed further that pod hair is a factor in *Vigna vexillata* resistance to the pod sucking bug both under the field and screenhouse conditions. IITA annual report and research highlights of 1987/88 (IITA, 1988) also recorded a high level of resistance to the *Maruca* pod borer and pod sucking bugs in hairy *V. vexillata* using fresh pods as test materials. The Pearson-correlation coefficient indicated linear relationships with strong correlation among the characters evaluated.

Table 1. Morphological evaluation of some qualitative characters of 390 accessions of both cultivated and wild cowpeas.

Traits	No. of cultivated accessions	No. of wild accessions	Total
Presence of pigment on stem			
Purple pigment	31	38	69
%		11.92	2923
Presence of stripes on pods			
Red stripes	51(19.61%)	2	53
Brown stripes	3 (1.15%)	0	3
%	20.76	1.53	
Plant habit			
Erect	19(7.3%)	1(0.77%)	20
Suberect	18(6.92%)	6(4.62%)	24
Prostrate	229(88.07%)	117(90%)	346
Hairiness on:			
stem, leaf and pod	0	14(10.77%)	14
Stem only	0	12(9.23%)	12

The Pearson-correlation coefficient was used to indicate the linear relationship between the characters (Table 2). A strong correlation was observed among the traits under study, and the number of traits for principal component analysis was reduced to only nineteen traits. This is because highly correlated traits showed no variance and can lead to an unreliable estimate of the variance in the principal components. Based on this, the first component (Prin 1) accounted for 60% of the total variation. The floral characters such as keel width (kwt), standard length (slt), adelphous stamen length, standard width (swt) and style length (stlt) were positively and highly correlated to the wing width (wwt), i.e., for a high wing width value, the other flower characters had a high value too, and contributed greatly to the diversity observed among the accessions (Table 2). A similar high and positive correlation was observed between wing length (wlt) and standard width (swt) and

wing width (wwt), keel width (kwt) with adelphous stamen length (aslt) and standard length (slt), adelphous stamen length (aslt) with single stamen length (sslt) and style length (stlt), and standard width (swt) and adelphous stamen length (aslt).

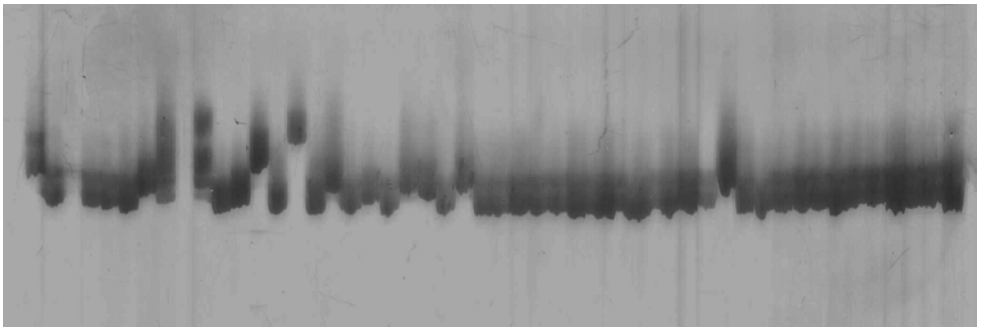
Table 2. The Pearson-correlation matrix to two decimal places of nineteen morphological characters evaluated for 390 accessions of cultivated and wild cowpeas.

	clt	cwt	clllt	cllt	wlt	wwt	klt	kwt	slt	swt	aslt	sslt	stlt	tlwt	tllt	pllt	plt	rlt	pult
clt	1	0.45	0.19	0.4	0.25	0.26	0.12	0.23	0.27	0.3	0.26	0.21	0.12	0.19	0.22	0.22	0.2	0.18	0.17
cwt		1	0.35	0.65	0.52	0.5	0.26	0.49	0.56	0.61	0.5	0.44	0.34	0.37	0.41	0.42	0.37	0.36	0.33
clllt			1	0.64	0.3	0.28	0.15	0.27	0.24	0.29	0.29	0.31	0.22	0.11	0.19	0.14	0.16	0.12	0.06
cllt				1	0.39	0.38	0.18	0.33	0.36	0.42	0.34	0.35	0.23	0.26	0.36	0.32	0.36	0.3	0.23
wlt					1	0.83	0.43	0.69	0.73	0.85	0.73	0.67	0.72	0.01	0.18	0.02	0.06	0.08	0.03
wwt						1	0.42	0.8	0.78	0.85	0.76	0.69	0.72	0.05	0.19	0.04	0.07	0.09	0.04
klt							1	0.48	0.36	0.41	0.44	0.38	0.41	0.01	0. 08	-0.01	0.02	0.02	0.02
kwt								1	0.68	0.71	0.76	0.66	0.65	0.06	0.17	0.04	0.09	0.09	0.07
slt									1	0.84	0.72	0.64	0.63	0.16	0.27	0.17	0.15	0.15	0.14
swt										1	0.77	0.69	0.74	0.09	0.23	0.1	0.12	0.14	0.09
aslt											1	0.8	0.87	0.08	0.23	0.06	0.07	0.09	0.08
sslt												1	0.79	0.03	0.18	0.00	0.01	0.03	0.05
stlt													1	-0.11	0.07	-0.14	-0.11	-0.05	-0.09
tlwt														1	0.49	0.68	0.65	0.56	0.65
tllt															1	0.56	0.53	0.45	0.51
pllt																1	0.8	0.72	0.72
plt																	1	0.82	0.59
rlt																		1	0.52
pult																			1

The characters are abbreviated as: calyx length (clt); calyx width (cwt); calyx lobe lt (cllt); total calyx lt (tclt) ; wing lt (wlt); wing width (wwt); keel lt (klt); keel width (kwt); standard lt (slt); standard width (swt); adelphous stamen lt (aslt); single stamen lt (sslt); style lt (stlt); terminal leaflet width (tlwt); terminal leaflet lt (tllt); petiolule lt (pllt); petiole lt (plt); rachis lt (rlt) and pulvin lt (pult).

Primers used varied in allele identification per accession in the study (Figure 1). A total of 92 resolved DNA bands were amplified by sixteen SSR primers in the 48 accessions evaluated. The distance matrices generated from genotyping data were used to construct the dendrogram (Figure 2). Three clusters were identified at a similarity coefficient of 82% that separated cultivated lines from their wild relatives. Clusters ‘A’ and ‘C’ consisted mainly of wild cowpea, while cluster ‘B’ comprised cultivated cowpea including accessions of subsp. *Sesquipedalis* and

subspp. *cylindrical*. Two *dekindtiana* (3 and 6), one *kgalagadensis* (10) and one *protracta* (20) wild accessions clustered with the cultivated cowpea. Accessions 13 and 14 were widely separated, and this may be attributed to leaf characteristics of these accessions. Their morphological evaluation revealed that accession Tvnu 1471 (14) (belonging to *rhomboidea)* had simple leaf characteristics as against the trifoliate leaf in *rhomboidea* accession Tvnu 1473 (13). Two accessions, Tvnu 533 and 538 (23 and 24) belonging to the ssp *pubescens* were also grouped separately from each other. Accession Tvnu 533 (23) did not cluster in either of the two clusters (A and B) while accession Tvnu 538 (24) was grouped in cluster 'A'. *Mensensis* (19) was observed as an outgroup to all the evaluated accessions in the study. *Mensensis* is a variety of subspecies *dekindtiana* with the long calyx lobe as against the short calyx lobe found in other wild cowpeas. This is a characteristic of *V. unguiculata* subspp. *dekindtiana* var. *mensensis*.



1 24 48

Figure 1. The electrophoregram of the combined accessions of cultivated and wild cowpeas with SSR primer VM 27. (Code: 1–24 = wild, 25–48 = cultivated with lanes 1–24 showing more segregation than lanes 25–48).

The information generated from the dendrogram showed that the cultivated cowpea was closely related to the wild species. This is important for understanding the taxonomy of the genus *Vigna*, the origin of cultivated cowpea and future cowpea breeding. Several hypotheses based on morphological attributes have been proposed about the origin of cultivated cowpea (Rawal, 1975; Lush, 1979; Steele and Mehra, 1980; Ng and Marechal, 1985). The wild annual subsp. *dekindtiana* has been suggested as the probable progenitor of the cultivated cowpea. Lush and Evans (1981) have also speculated that the subsp.subsp. *dekindtiana* is a more probable progenitor type than subsp.subsp. *mensensis* because it is morphologically closer to the cultivated cowpea. He stressed further that subsp. *dekindtiana* could have originated from hybrids of subsp. *mensensis* with subspecies *unguiculata*.

This corroborates with the work of Pasquet (1999), who reported subsp. *spontanea* as the closest variety to cultivated cowpea. It is also quite possible that what was classified as subsp. *spontanea* is also referred to as *dekindtiana*, i.e., the same subsp. Fatokun et al. (1993) used RFLP markers to demonstrate that *Vigna unguiculata* subsp. *unguiculata* var. *sesquipedalis* showed high phyletic relationships with cultivated cowpea and its wild relatives of subsp. *dekindtiana*. However, results from this work did not show a contrary opinion from past findings.

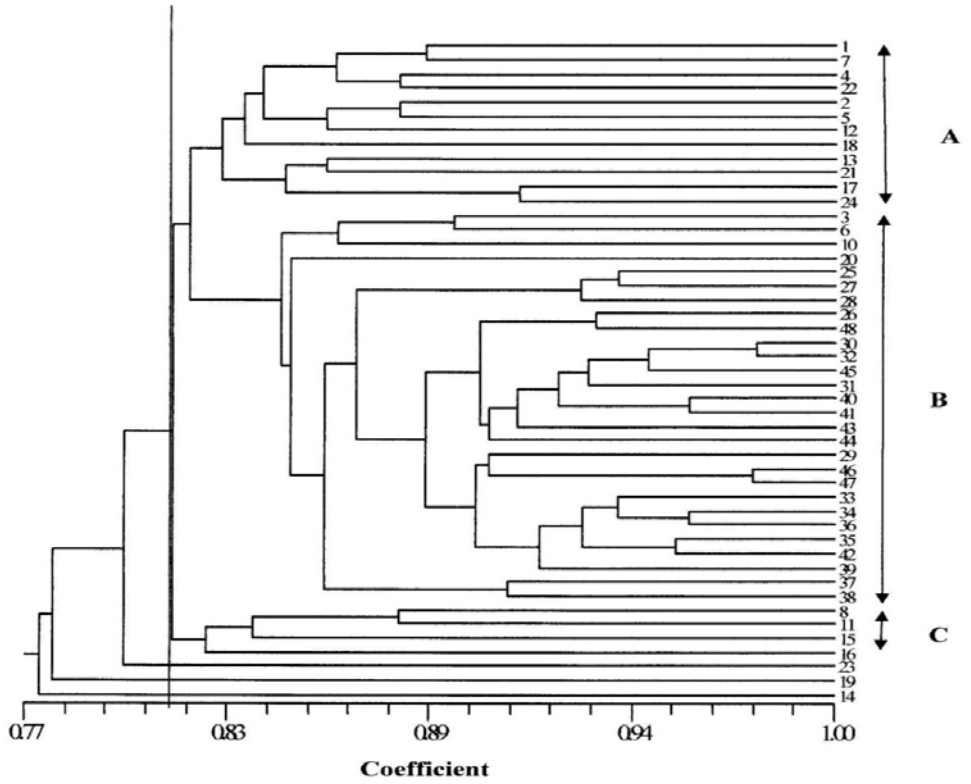


Figure 2. The UPGMA dendogram showing the clustering of forty-eight cowpea accessions (cultivated and wild) using sixteen SSR primers. The codes indicate the types of cowpea used: 1 – 9 *dekindtiana*, 10 – *kgalagadensis*, 11 and 12 – *grandiflora*, 13 and 14 – *rhomboidea*, 15–17 – *congolensis*, 18 – *ovata*, 19 – *mensensis*, 20 and 22 – *protracta*, 23 and 24 – *pubescens*, 25–45 – *unguiculata*, 46 and 47 – *sesquipedalis* and 48 – *cylindrical.*

The principal component analyses further clarified the relationships between cultivated and wild cowpeas. For molecular data, the first and the second principal

components (PCs) accounted for 67.45% and 3.75%, respectively, of the total variations in the correlation matrix, which was considered a good summary of the data. The two-dimensional plot (Figure 3) based on these two axes showed a clear pattern of differentiation although there was the overlapping of some wild accessions in the group of cultivated ones. For instance, two wild subspecies *dekindtiana* (3) and *protracta* (20) were grouped together with the cultivated accessions. Wild lines were distinctly separated from the cultivated group, especially *rhomboidea* and *mensensis* with Tvnu1471 (14) and Tvnu 1561 (19), respectively, indicating their distinctiveness as revealed by the UPGMA dendrogram. After examining the gels developed from the sixteen polymorphic primers for shared bands between wild and cultivated cowpeas, the results showed that *dekindtiana* had the highest percentage (80%) of shared bands with cultivated cowpea, followed by *protracta* at 54%, while others were *kgalagadensis* at 48%, *pubescens* at 40%, *congolensis* at 39%, *mensensis* at 36%, *ovata* at 28%, and *rhomboidea* at 27%. The high percentages obtained for both *dekindtiana* and *protracta* may be a result of their closeness to the cultivated cowpea, as close relatives are likely to have matching bands. The study also revealed a higher PIC value in wild cowpea (6.14) than in cultivated cowpea (4.47).

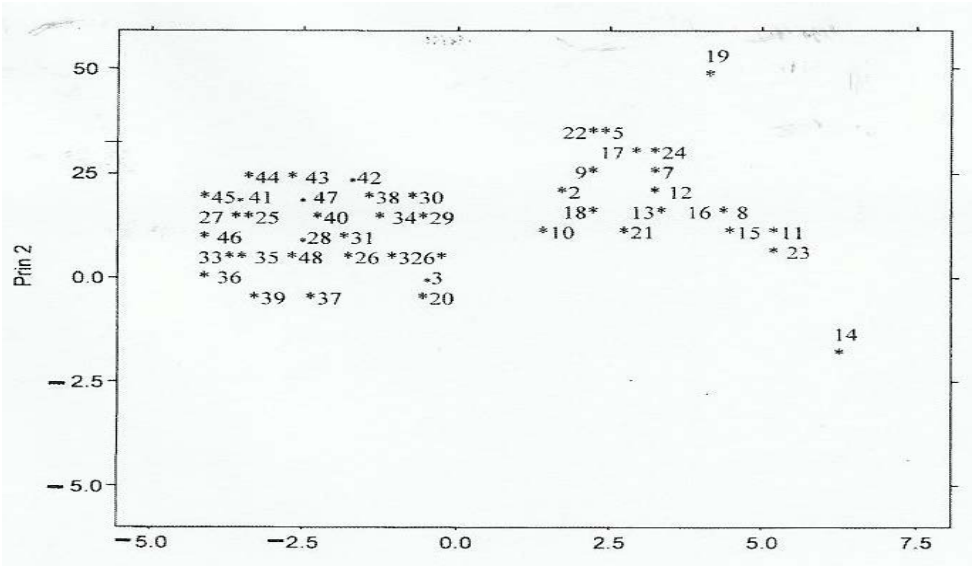


Figure 3. The principal component analysis of forty-eight accessions of cowpea (cultivated and wild) using SSR primers, (1–24 are wild, and 25–48 are cultivated cowpeas).

From the study, both *dekindtiana* and *protracta* consistently showed good relationships with the cultivated cowpea and clustered together. This is an indication of genetic relatedness with cultivated cowpea and probable domestication of these wild relatives. This suggests that breeders could use these two wild relatives to improve the cultivated cowpea with the intent of introgressing useful traits into cultivated cowpea. Studies have confirmed the cross-compatibility between cultivated and wild cowpeas, especially the *dekindtiana*.

The polymorphic information content (PIC) value for cultivated cowpea was 4.467 from a total of thirty-eight alleles detected from sixteen SSR primers. This high value was in agreement with the results obtained in past works (Ali et al., 2015) although this value was lower than those reported in other legumes, like *P. vulgaris* (Singh et al., 1991) and some other non-legumes like rice (Second, 1985). This could be because cowpea was domesticated at only one place. However, wild cowpea had a higher PIC value of 6.136 from fifty-four alleles with the same number of SSR primers. The estimates of PIC and allele frequency from this study indicated that wild cowpea was much more genetically diverse than cultivated cowpea. We also observed lower genetic variation within cultivated cowpea compared to the wild accessions, probably due to wider geographic distribution of the wild species, with *rhomboidea* (14) and *mensensis* (19) far apart from remaining accessions.

Conclusion

The study revealed a diverse morphological trait between cultivated and wild cowpeas evaluated in the study, with hairiness only found on wild cowpea pods and stems. The molecular evaluation showed wild cowpea lines were distinctly separated from the cultivated group, especially *rhomboidea* and *mensensis.* However, both morphological trait evaluation and molecular data showed lower genetic variability between both cultivated and wild cowpea species evaluated in the study although widely distributed geographically.

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MORFOLOŠKA KARAKTERIZACIJA I KARAKTERIZACIJA MARKEROM SSR DIVLJIH I UZGAJANIH VIGNI (*VIGNA UNGUICULATA* L. WALP)

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R e z i m e

Tristadevedeset genotipova koji su obuhvatali 260 gajenih i 130 genotipova divlje vigne fenotipski su ocenjivani korišćenjem 27 deskriptora vigne. Morfološka evaluacija nekih kvalitativnih osobina otkrila je od 11,92% do 29,23% prisustvo pigmentacije na stabljici, 1,53% do 20,76% prisustvo pruga na mahuni i 0% do 20% prisustvo dlakavosti na biljci gajene odnosno divlje vigne. U pogledu molekularne analize, šesnaest SSR prajmera su korišćeni za genotipizaciju 48 genotipova koji pripadaju kako divljoj tako i gajenoj vigni. Podaci su dali dendogram sa tri klastera, od kojih su se dva sastojala od divlje vigne dok je treći obuhvatao sve gajene vigne, uključujući i genotipove sa dugačkom mahunom (*Vigna unguiculata* subsp. *sesquipedalis)* i *Vigna unguiculata* podvrste *cylindrica*. Dva divlja genotipa podvrste *dekindtiana,* i po jedan iz podvrsta *kgalagadensis* i *protracta* grupisali su se sa genotipovima gajenih vigni ukazujući na njihove veze, ali ne i sa drugim divljim vignama. Broj polimorfnih traka SSR kod gajenih i divljih vigni bio je 38 odnosno 54, dok su vrednosti PIC bile 4,47 odnosno 6,14, što pokazuje veću genetsku raznovrsnost kod divljih nego kod gajenih vigni. Podvrsta *dekindtiana* imala je najveći broj (80%) prikazanih traka primenom SSR markera sa gajenom vignom, a zatim je sledila podvrsta *protracta* sa 54% prikazanih traka. Pet vrsta divlje vigne ima dlake, te bi se tako mogle koristiti u oplemenivanju za otpornost prema insektima.

Ključne reči: vigna, karakterizacija, dendrogram, genetska raznovrsnost, mikrosateliti.

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SEED PERFORMANCE OF COMMON BEAN AND COWPEA BY PRIMING AND PLANTING DATE

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Abstract: A 2-year farm trial by employing the nutrient priming technique on common bean (Sarıkız) and cowpea (Şimal) seeds was performed to assess seed yield and protein content. Priming: (p1) control, (p2) KH_2PO_4, (p3) $ZnSO_4$ and (p4) KH_2PO_4+$ZnSO_4$, planting date: (d1) 20 May, (d2) 15 June 2015, (d1) 7 May, (d2) 7 June 2016 were used to study the yield (kg ha^{-1}) and crude protein (%) of the mentioned crops in Ankara, Turkey. In 2015, application of p3 and p4 (868.8, 834.8), d2 (962.3) and p4d2, p2d2 and p3d2 (1061, 1052, 1028) increased seed yield of Sarıkız, and p2 and p1 (899.5, 835.9) and d2 (955.9) increased the yield of Şimal. Treatments of p4 and p3 (18.7, 18.5), d2 (19.2), and p4d2 (21.3) increased the protein of Sarıkız and d2 (19.4) increased the protein of Şimal. In 2016, p3 (2506), d2 (2516) and p3d2 (3650) increased the yield, and p4 (26.1), d2 (26.8) and p4d2 (28.3) increased the protein of Sarıkız. Treatments of p3 (1979.1), d2 (2664.3) and p3d2 (3310.6) increased the yield, and d1 (24.1) and p3d1 (25.7) increased the protein of Şimal. Application of Zn and P by seed priming seems to effectively increase the yield and protein content of these crops.

Key words: common bean, cowpea, crude protein, planting date, priming, yield.

Introduction

The most effective way to alleviate phosphorus (P) deficiency in crops is to add large quantities of phosphate fertilizers to the P-deficient soils, either as P

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solutions or soil applications. However, amelioration of P deficiency with large amounts of fertilizers is not a viable option for many farmers. Other methods for providing seeds with nutrients have shown serious limitations. Making high concentrations of P in seeds by increasing soil fertilization would increase the cost of seed production, as a result of partial fixation of this nutrient in the soil. Another way of providing seeds with P is coating them with this element which can stimulate early plant growth, but is less effective in increasing grain yield of plants, such as wheat in the field (Peltonen-Sainio et al., 2006). However, seed dressing of some nutrients has been advised as a low-cost and highly effective approach, but priming the seeds with small amounts of nutrients before planting has been shown to partially increase nutrient use efficiency and it has been found to be effective for legume yield, so that a considerable increase in their yield has been observed (Musa et al., 2001; Rashid et al., 2004; Harris et al., 2004). Seed P content, although constitutes a small proportion of total plant demand, may be effective for optimum plant establishment and growth. Generally, seed priming with P and zinc (Zn) nutrients around or within the seed may be an attractive solution to overcome P and Zn deficiencies and it has been found that on-farm seed priming with solutions of KH_2PO_4 increased the yield of different crops grown on P-deficient soils (Ali et al., 2008). Some investigations have been performed using primed seeds of chickpea and wheat (Harris et al., 2004) and maize (Harris et al., 2007) with Zn solutions leading to high seed yields.

In terms of protein content of seed legumes, there are few studies on seed priming with Zn and P elements in spite of findings on the impacts of these on the improvement of the protein content of the cowpea (Chavan et al., 2012,) common bean (Poshtmasari, 2008) and green gram seeds (Muhammad et al., 2014). Also, reports on the combined application of foliar spraying along with seed priming with Zn containing solutions in crops such as black gram which has increased protein content are found in the literature (Kshama, 2013).

Planting date as a climatic factor plays an important role in the final yield of legumes by affecting the vegetative and reproductive stages and their ratio (Lopez-Bellido et al., 2008). Suitable planting date causes the flowering stage not to coincide with high temperatures, leading to the highest pod production. Also, suitable planting date prolongs the duration of the vegetative stage to transport more assimilates to the sinks while the late planting date results in the early flowering leading to a dry matter reduction, a decline in the produced pods and finally yield loss (Mozumber et al., 2003). It has been suggested that early cultivation of warm-season plants may negatively affect preliminary seedling establishment due to the lower rates of air temperatures (onset of spring). Also, when planting is performed at the beginning of the season which is earlier than optimum date, seed yield loss in such plants is expected because of encountering the stages of flowering, anthesis and early seed filling with high temperatures of

July and August. On the other hand, late planting may prolong the seed filling period due to the lower air temperatures of the end of summer and early autumn (Khajehpour, 2000). In this regard, it has been shown that in the semi-arid conditions of Northern Iran, high yields of cowpea have been obtained in June cultivation (Farahmand-Rad, 1999).

The objective of this work was to assess the quality and quantity responses of common bean and cowpea plants to phosphorus and zinc seed treatments grown at different planting dates.

Materials and Methods

The field trial was run at the research farm of the Faculty of Agriculture, Ankara University, Ankara, Turkey during 2015 and 2016 growing years for the planting of the primed seeds to measure the seed yield of the common bean (*Phaseolus vulgaris*, Sarıkız), and cowpea (*Vigna unguiculata*, Şimal) crops. The priming section of the present work was carried out in the Laboratory of Seed Sciences, Faculty of Agriculture, Ankara University, Ankara, Turkey. For measuring the crude protein content of the crop, harvested seeds were analyzed in the Laboratory of the Soil and Plant Nutrition, Faculty of Agriculture, Ankara University. This work was carried out as a factorial experiment based on a randomized complete block design (RCBD) with three replicates.

Table 1. Different dates of planting, flowering and harvesting of the Sarıkız and Şimal crops in 2015 and 2016.

	Planting	Flowering	Harvesting
2015 – the first date			
Sarıkız	20/05/2015	28/06/2015	08/08/2015
Şimal	20/05/2015	15/07/2015	15/08/2015
2015 – the second date			
Sarıkız	15/06/2015	22/07/2015	05/09/2015
Şimal	15/06/2015	02/08/2015	05/09/2015
2016 – the first date			
Sarıkız	07/05/2016	22/06/2016	01/08/2016
Şimal	07/05/2016	14/07/2016	10/08/2016
2016 – the second date			
Sarıkız	07/06/2016	14/07/2016	22/09/2016
Şimal	07/06/2016	30/07/2016	13/09/2016

Factors included different seed priming treatments including (p1) control (untreated), (p2) KH_2PO_4 10 g L^{-1}, (p3) $ZnSO_4$ 4 g L^{-1} and (p4) KH_2PO_4 5 g L^{-1}+$ZnSO_4$ 2 g L^{-1} and planting dates: (d1) 20 May and (d2) 15 June for the year of 2015, and (d1) 7 May and (d2) 7 June for the year of 2016. In both years, before

planting, seed priming was performed for 7 hours by immersion of seeds inside the mentioned salt solutions followed by drying of the seeds for 36 hours at 25°C and thereafter, the seeds were subjected to planting. Each plot comprised 5 rows three meters long and 50 cm apart, seeds were planted manually in the rows with a distance of 10 cm between the two plants.

For weed controlling in the first year, hand hoeing was employed for three times over the season. In the second year, Treflan® (Trifluralin) herbicide was applied on the soil surface and incorporated to the soil by a rotary tiller one week before planting.

Irrigation was done after planting and continued in 7–10-day intervals depending on the rainfall of the season and climatic conditions. At the end of the growing period of each planting date, while the pods turned yellow-gray in color, seeds were harvested from the two middle rows of each plot manually.

Seed yield

Sampling for measuring the seed yield was performed for all plants of the two middle rows of each plot by clipping them at the ground level including the whole pods. Since some of the pods were not absolutely dry while being at the maturity, they were left for air drying in plots for two days. Nearly at 13% relative humidity of the harvested seeds, pods were threshed to obtain the seeds and weighed as the yield per plot and then calculated as kg ha^{-1}.

Seed crude protein content

Seed crude protein content was measured for the harvested seeds. The amount of 0.5g of each seed set concerning the relevant priming treatment was taken. Samples were ground to fine flour for about 30 seconds and from which 0.252 g was stored at -20 °C until the protein extraction. Flour samples were subjected to determine nitrogen content by the micro Kjeldahl (AOAC, 2000) method and crude protein content was calculated by multiplying nitrogen % values by a conversion factor of 6.25.

Also, monthly average temperatures and monthly maximum temperatures over the growing seasons of the years of 2015 and 2016 in the experimental site (Ankara, Turkey) have been demonstrated in Table 2.

Statistical analysis

Data were subjected to analysis by MSTAT-C program, mean comparisons were performed by Duncan's multiple range test at $P < 0.05$, and graphs were drawn by Excel software.

Table 2. Monthly temperatures and monthly maximum temperatures (°C) of the years of 2015 and 2016 in Ankara, Turkey.

Months	May	June	July	Aug	Sep
2015					
Monthly average temperature (˚C)	16.6	20.2	25.9	26.0	19.4
Monthly maximum temperature (˚C)	28.9	34.1	36.5	38.1	34.9
2016					
Monthly average temperature (˚C)	17.2	18.4	24.9	24.7	23.4
Monthly maximum temperature (˚C)	32.1	28.1	38.0	34.7	34.6

Results and discussion

Data of the analysis of variance of the seed yield and crude protein influenced by the priming and planting date of the years of 2015 and 2016 has been shown in Table 3.

According to the results of the analysis of variance for both years and crops, effects of priming, planting date and interaction of priming and planting date (except for the interaction observed for Şimal in 2015) were significant (Table 3).

Table 3. Analysis of variance for the mean of squares (MS) values of studied traits: seed yield and seed crude protein content as affected by priming and planting date factors on Sarıkız and Şimal crops for 2015 and 2016 growing years.

Source	df	MS (2015)		MS (2016)	
		Yield	Crude protein	Yield	Crude protein
(Sarikiz)					
Replication	2	73.9	0.230	119918.1	6.246
Priming	3	74979.2*	2.726*	606898.4*	3.932*
Planting date	1	905088.5*	37.951*	4731797.0*	70.384*
Priming x Planting date	3	47770.9*	8.166*	1894849.6*	6.417*
(Şigmal)					
Replication	2	41877.4	4.451	60332.7	3.631
Priming	3	118372.4*	0.629^{ns}	724647.3*	1.004^{ns}
Planting date	1	983947.5*	140.796*	23660608.6*	37.026*
Priming x Planting date	3	40811.0^{ns}	2.610^{ns}	534176.8*	3.264*

*Significant at $P < 0.05$; ns Not significant.

Planting date resulted in the significant difference between the yields of the two planting dates so that the second one caused 962.3 kg ha^{-1} of yield (Figure 1e). The rate of the seed yield at the first planting date reached 573.9 kg ha^{-1}, which demonstrates a nearly1.5-fold decrease compared to the second planting date. Also, p4d2, p2d2 and p3d2 interactions (1061, 1052 and 1028 kg ha^{-1}, respectively) jointly resulted in the highest seed yields (Figure 1f).

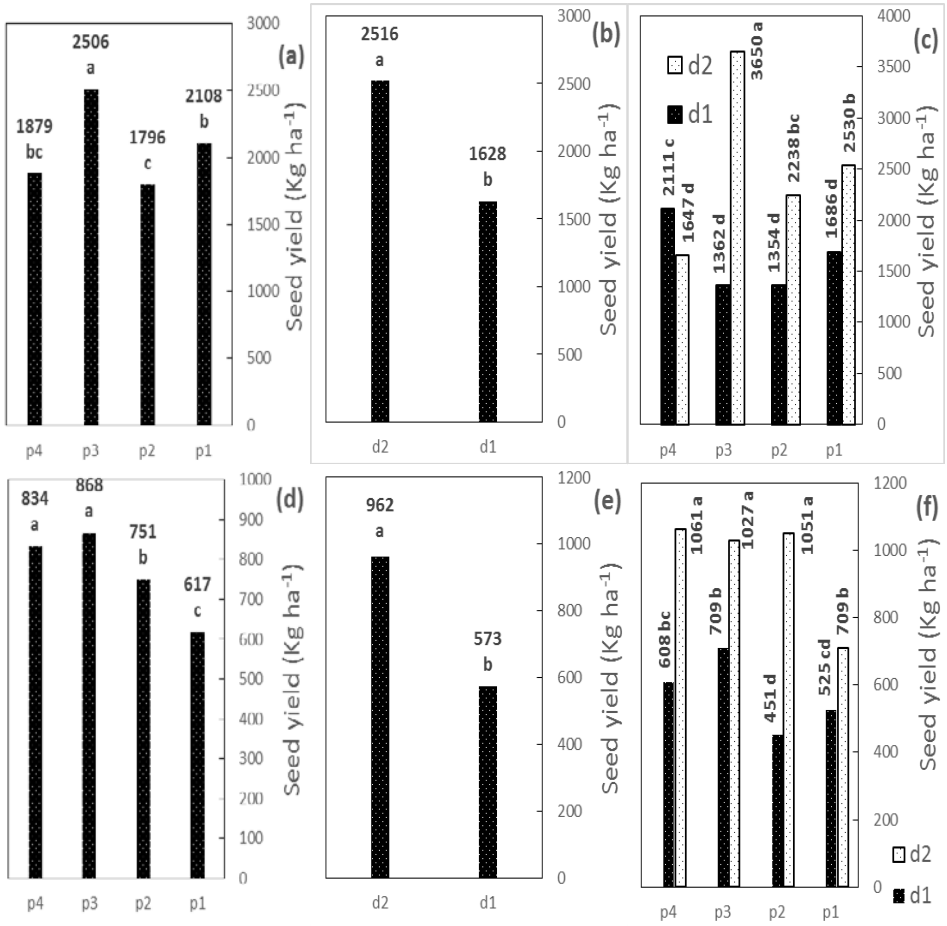

Figure 1. The impact of different priming solutions (p1, p2, p3, p4) and planting dates (d1, d2) on the yield of *Phaseolus vulgaris*, Sarıkız, including priming (a), planting date (b) and interaction of priming × planting date (c) for the year of 2016, and priming (d), planting date (e) and priming × planting date (f) for the year of 2015, in the order of the highest rates. Data with the same letters have no significant differences to each other at $P < 0.05$.

Similar to the year of 2015, the second planting date caused the highest yield (2516 kg ha^{-1}) (Figure 1b). Furthermore, the interaction of planting date and priming (p3d2) was significantly effective on the yield (3650 kg ha^{-1}) (Figure 1c). For Şimal, the same to the results of Sarıkız, the p3 treatment and the second planting date were significantly effective on the yield (1979.1 and 2664.3 kg ha^{-1}, respectively) (Figures 2a and 2b). As shown in Sarıkız, the interaction of priming

and planting date resulted in the highest seed yield (p3d2 with 3310.6 kg ha^{-1}) as well (Figure 2c). Similar to Sarıkız, the planting date showed a significant difference in terms of seed yield of Şimal and the highest rates obtained at the second planting date (955.9 kg ha^{-1}) (Figure 2e).

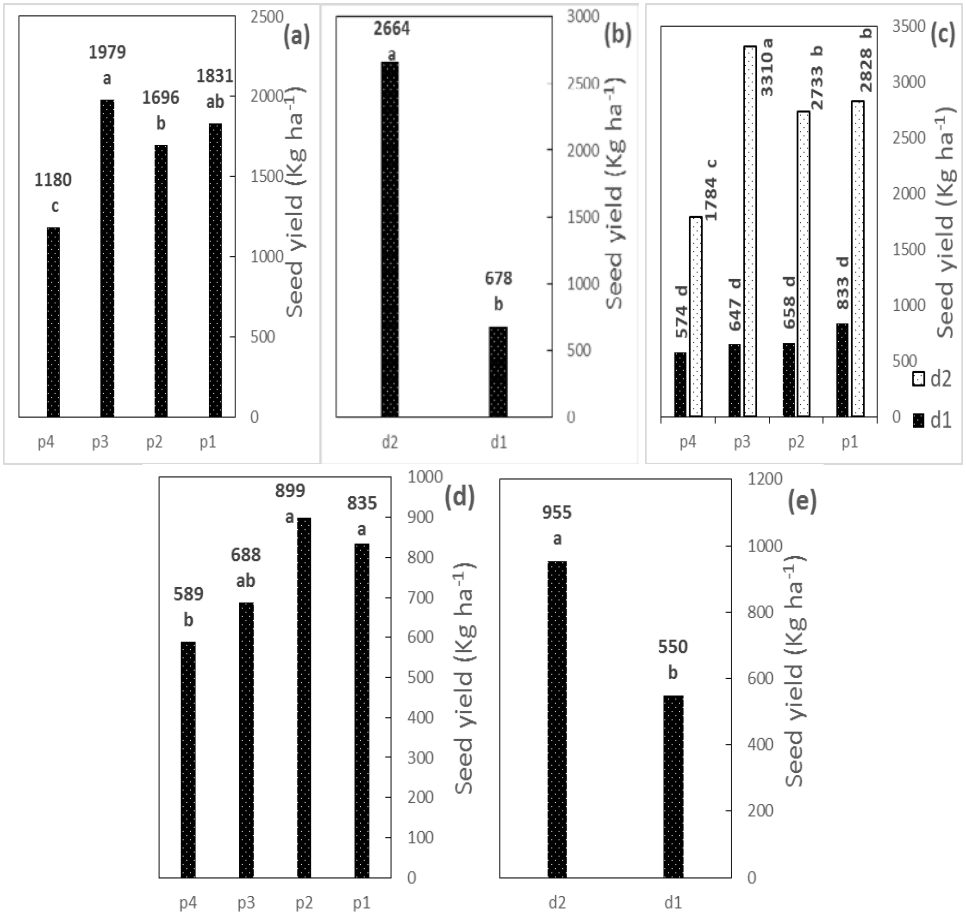

Figure 2. The impact of different priming solutions (p1, p2, p3, p4) and planting dates (d1, d2) on the yield of *Vigna unguiculata*, Şimal, including priming (a), planting date (b) and interaction of priming × planting date (c) for the year of 2016, and priming (d) and planting date (e) for the year of 2015, in the order of the highest rates. Data with the same letters have no significant differences to each other at $P < 0.05$.

At the second planting date of the first year (2015), in relation to the first planting date, the growth period of Sarıkız from the flowering to the harvest time lasted 4 days more (45 compared to 41 days for the first date). In the same way, the growth period of Şimal from flowering to the harvest time lasted 34 days, which is 3 days more than that of the first date. For the second year (2016), these rates for the first and second planting dates of Sarıkız were 40 days versus 70 (30 days more than the fırst date) and these of Şimal were 27 and 45 days (18 days more than the first date). As we can see, in both years, the second planting dates showed the longer time to the harvest, leading to higher yields than those of the first dates most likely due to the more time to produce higher dry matter as affected by the climatic conditions, and especially air temperatures (Table 2) and this is the main reason for remarkably higher yields of the year of 2016 for both crops.

Optimum temperatures for bean crop vary between 21 and 25°C depending on the growth phase (Ferreira et al., 1997) and despite being warm-season crops, both Sarıkız and Şimal were negatively affected by high temperatures, especially over the reproductive stage. Considering Table 2, it is clear that July and August were the warmest months across the growing seasons of both years and it should be noted that the planted crops at the first date suffered from the high temperatures over July. For example, the first planting date of the second year with longer exposure to the high temperatures resulted in the lowest amounts of the pods/plant for both crops, as a yield component (data not shown), which is in accordance with the findings of Adam et al. (2013). This decrease in pod number may be attributed to the failure of pollination which has been shown in kidney bean (Prasad et al., 2002). In addition, it has been reported that rising air temperature from 29 to 34°C during the seed filling stage significantly decreases soybean seed yield (Dornbos and Mullen, 1991; Adam et al., 2013). Furthermore, it has been demonstrated that temperatures above 30/25°C (day/night) during the flowering and pod development reduce seed weight, regardless of temperatures during the seed filling period (Egli and Wardlaw, 1980). As being the yield components, the number of seeds per pod is negatively affected by high temperatures as well (Baker et al., 1989). All of these features indicate the negative impact of high temperatures, which mostly coincides with the flowering stage, on the crop yield. In this regard, other researchers (Mani and Abas, 2014) have reported a decrease in the yield of the cowpea planted at the early season of the warm regions of Iran, which confirms our results on the yield loss resulted from the first planting date.

In the year of 2015, p3 and p4 treatments jointly increased the seed yield of Sarıkız at rates of 868.8 and 834.8 kg ha^{-1} compared to the control and p2 treatments (Figure 1d). Contrary to the Sarıkız, p3 and p4 treatments did not affect the seed yield in Şimal. In the recent crop, p2 and p1 increased the yields at rates of 899.5 and 835.9 kg ha^{-1}, respectively (Figure 2d). Hence, seed priming with KH_2PO_4 was less effective on the yield of Şimal, the same way it was demonstrated

in Sarıkız. Furthermore, another anomaly, compared to Sarıkız, is the remarkable impact of the non-primed seeds of Şimal on the yield increment.

In the year of 2016, p3 priming of Sarıkız resulted in the highest seed yield of 2506 kg ha^{-1} (Figure 1a). Treatments of p4 and p1 were classified in the same group.

We found that providing the seed with P can be as important as planting date for improving the seed yield. There are some findings indicating the role of seed content of P in increasing grain yield of lupins (Bolland et al., 1989), pasture legumes (Bolland and Paynter, 1990), wheat (Burnett et al., 1997) and maize (Miraj et al., 2013) enriched with P (not merely by the priming procedure) under the field conditions. Although seed soaking in P solutions may delay seed germination of common bean (Teixeira et al., 1999), several reports indicate the benefits of P supplied by the priming on the seed yield of bean (Setareh et al., 2013; Sixbert and George, 2012; Adelson et al., 2000) and cowpea (Ntare and Bationo, 1992; Nkaa et al., 2014). Also, soaking seeds of barley (Zhang et al., 1998) and mung bean (Adnan et al., 2011) in P solutions has improved their yield that is in accordance with our results on Şimal and Sarıkız in the first year. Like P, seed treatment with Zn shows beneficial aspects in primed seeds. This element (Zn) is required for plant growth and is directly involved in the biosynthesis of growth regulators needed for the production of cells and biomass that will be stored in the plant organs, especially in seeds (Marschner, 1995). It has been shown that seed priming of rice with Zn resulted in higher uptake of this element by the seedling (Nathan et al., 2001), which may cause the yield improvement. Also, an increase in Zn content and final yield of chickpea primed with $ZnSO_4$ has been observed (Harris et al., 2008). In addition, it has been reported that seed priming with Zn solutions significantly improved yield and related traits in common bean (Kaya et al., 2007) and chickpea (Mahnaz et al., 2015). These findings are in accordance with our results.

According to the results of both years for Sarıkız and Şimal (Table 3), priming, planting date and their interactions were significantly effective on the seed protein content except for priming in the second year for both crops.

In the year of 2015, the application of p4 and p3 on Sarıkız jointly increased the seed protein content compared to the control and p2 treatments, at rates of 18.7 and 18.5%, respectively (Figure 3d).

Also, the second planting date remarkably increased the seed protein content of 19.2% (Figure 3e), and p4d2 interaction significantly increased the seed protein content (21.3%) (Figure 3f). Contrary to Sarıkız, the application of different seed priming treatments did not lead to any crude protein improvement and only planting date was effective on the seed protein content (19.4%) (Figure 4c).

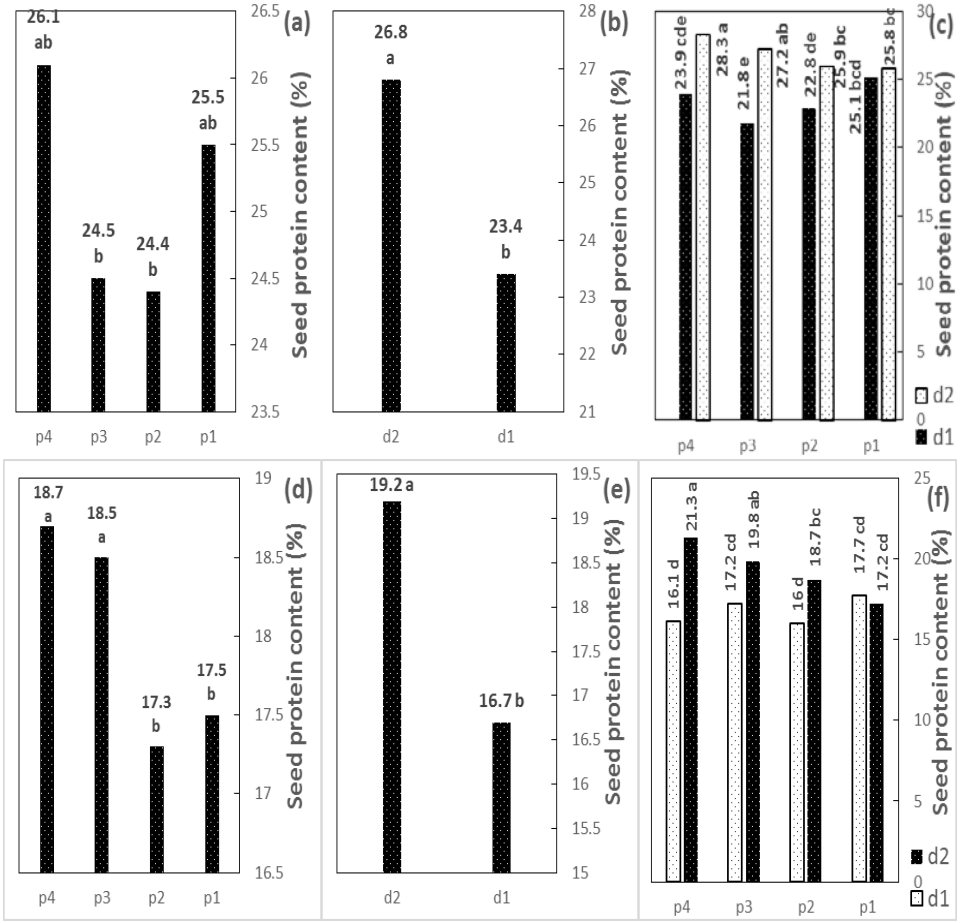

Figure 3. The impact of different priming solutions (p1, p2, p3, p4) and planting dates (d1, d2) on the crude protein content of *Phaseolus vulgaris*, Sarıkız, including priming (a), planting date (b) and interaction of priming × planting date (c) for the year of 2016, and priming (d), planting date (e) and priming × planting date (f) for the year of 2015, in the order of the highest rates. Data with the same letters have no significant differences to each other at $P < 0.05$.

In the year of 2016, the same to the results of Sarıkız for the year of 2015, the crude protein content of Sarıkız was significantly increased using p4 treatment (26.1%), but the effect of p3 was not significant on this trait (Figure 3a). Also, the second planting date was significantly effective on the crude protein content of Sarıkız in both years and the highest amount (26.8%) (Figure 3b) was achieved in the second year (7% more protein content). In addition, p4d2 interaction

significantly yielded the highest protein content of 28.3% (Figure 3c) in 2016 and 21.3% in 2015 applied on Sarıkız. Considering the effect of planting date on Şimal, it is observed that contrary to the year of 2015, the first planting date of 2016 significantly improved the crude protein content (24.1%) (Figure 4a). Finally, the interaction of p3d1 increasingly raised the crude protein content compared to the other combinations (25.7%) (Figure 4b). With regard to the findings of both years, it is clear that the year of 2016 had the relatively higher values both in terms of seed yield and seed crude protein content and this difference in the measured traits was higher, especially in values of the year of 2016.

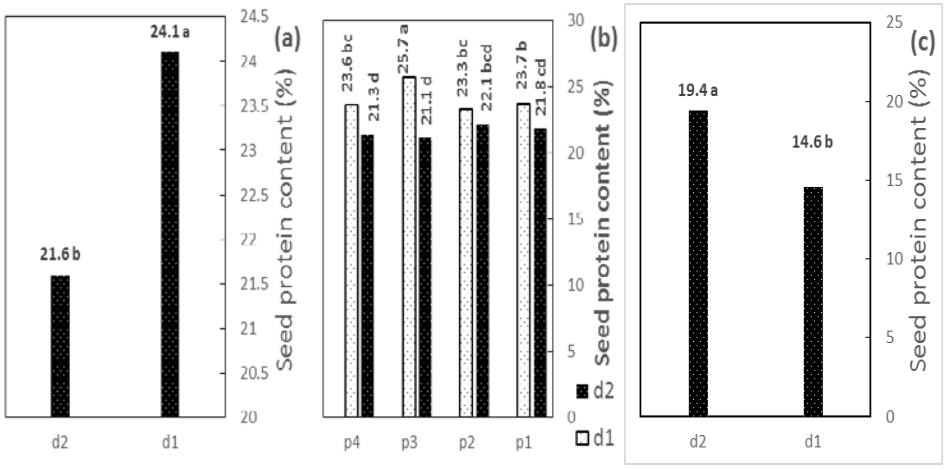

Figure 4. The impact of different priming solutions (p1, p2, p3, p4) and planting dates (d1, d2) on the crude protein content of *Vigna unguiculata*, Şimal, including planting date (a) and interaction of priming × planting date (b) for the year of 2016, and planting date (c) for the year of 2015, in the order of the highest rates. Data with the same letters have no significant differences to each other at $P < 0.05$.

There are few studies on supplying Zn for increasing the protein content of legumes by seed priming while the relationship between seed Zn content and protein concentration in cereal and legume seeds has been found (Levent et al., 2006; Cakmak et al., 2004). Priming is considered as one of the ways to enhance seed content of Zn. In this regard, Johnson et al. (2005) found that Zn content of chickpea and lentil seeds after priming with $ZnSO_4$ solution increased about 10-fold in comparison to the initial Zn content. These rates may also be provided by the other fertilization methods but without any benefits of the priming. Zn actively affects nitrogen metabolism that may result in protein synthesis (Fageria et al., 2003). In other words, protein synthesis is negatively affected by Zn deficiency, and an increase in the seed protein content makes a sink for Zn, and there is a close positive correlation between the seed protein content and Zn concentration

(Cakmak et al., 2010). It has been found that in case of higher rates of Zn concentration in shoot meristematic tissues of rice than in mature leaves, a high rate of protein synthesis is observable (Kitagishi and Obata, 1986), while in leaves of bean under conditions of Zn deficiency, the concentration of soluble proteins is decreased (Cakmak, 1989). Also, a significant positive effect of zinc treatment on crude protein content in the seeds of mung bean has been found (Krishna, 1995). Various enzymes are responsible for the carbohydrate metabolism and protein biosynthesis and activation and kinetics of these enzymes intrinsically depend on Zn content (Fageria et al., 2003; Broadley et al., 2007). Similar to the seed priming, other methods of Zn application like foliar spraying increase the seed protein content of crops such as cowpea (Hemn, 2013). In addition to the Zn, some studies have shown that P nutrient has led to the increase in the seed protein content of soybean (Dalshad et al., 2013; Soares et al., 2014), rice bean (Asghar Malik et al., 2002) and moth bean (Nishi et al., 2007). This may be due to the improved uptake of elements like N in plants subjected to the seed priming with KH_2PO_4. In such cases, an increase in the protein content of 21-day-old mung bean plants has been observed (Adnan et al., 2013). Also, the seed treatment of mung bean with KH_2PO_4 enhances nitrogen fixation (Adnan et al., 2011). High seed P concentration produces plants less dependent on soil P supply and can enhance nitrogen fixation in common bean which leads to the higher protein content of the seeds (Teixeira et al., 1999). In addition, P is necessary for seed formation and is a fundamental element for nodule metabolism in legumes (Gutierrez-Rodriguez et al., 2006) and it is required to regulate the activity of several proteins through phosphorylation reactions (Martinez-Ballesta et al., 2010).

Conclusion

Planting date significantly influenced seed protein content in Sarıkız in 2015 and 2016 and this result was obtained in Şimal at least in 2015. In terms of seed yield, the same trend was observed in Sarıkız and Şimal in the second planting date of 2015 and 2016 indicating that the planting date of these plants should be arranged so that the flowering stage does not coincide with the warmest temperatures of the summer which may be achieved by few days of delay in the early spring. Also, considering the findings of this work on the impact of P and Zn priming on an increase in the seed yield and protein content, we suggest that by increasing P, as an element consumed in legumes in large amounts, and Zn content of the seeds of Sarıkız and Şimal, high rates of yield and protein content may be achievable while cellular mechanisms and changes in the plant metabolism linked to the priming remain unknown to some extent, and we propose further studies in this regard.

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UTICAJ PREDSETVENOG NATAPANJA SEMENA I MOMENTA SETVE KOD PASULJA I CRNOOKICE

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R e z i m e

Ispitivan je uticaj unošenja hranljivih materija potapanjem semena pre setve (p1- kontrola, p2 - KH_2PO_4, p3 - $ZnSO_4$ i p4 - KH_2PO_4+$ZnSO_4$) i datuma setve kod pasulja (sorta Sarıkız) i crnookice (sorta Şimal) na prinos zrna (kg ha^{-1}) i sadržaj belančevina (%) u njemu. Ogled je izveden na otvorenom polju u Ankari (Turska) tokom dve godine. Datumi setve u 2015. godini bili su d1 - 20. maj, d2 - 15. jun, a 2016. godine d1- 7. maj i d2 - 7. jun. U 2015. godini, primenom tretmana p3 i p4 (868,8, 834,8) setvom u junu (962,3), kao i međusobnim uticajem p4d2, p2d2 i p3d2 (1061, 1052, 1028) povećan je prinos semena pasulja. Tretmani p2 i p1 (899,5, 835,9) i junska setva (955,9) povećali su prinos crnookice. Tretmani p4 i p3 (18,7, 18,5), junska setva (19,2), i međusobni uticaj p4d2 (21,3) povećali su sadržaj proteina u zrnu pasulja. Junska setva (19,4) povećala je sadržaj proteina kod crnookice. U 2016. godini tretman p3 (2506), junska setva (2516) i kombinacija p3d2 (3650) povećali su prinos, a tretman p4 (26,1), junska setva (26,8) i kombinacija p4d2 (28,3) povećali su sadržaj proteina kod useva pasulja. Tretmani koji su uključivali p3 (1979,1), junska setva (2664,3) i njihova kombinacija, p3d2 ,(3310,6) povećali su prinos, a setva u maju, d1, (24,1) i njena kombinacija sa tretmanom P3, p3d1, (25,7) povećali su sadržaj proteina kod crnookice. Izgleda da primena Zn i P potapanjem efikasno povećava prinos i sadržaj proteina ovih useva.

Ključne reči: pasulj, crnokoica, sirovi protein, datum setve, potapanje semena, prinos.

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PROFILES OF COMPOUNDS IN ROOT EXUDATES OF RICE, CYMBOPOGON, DESMODIUM, MUCUNA AND MAIZE

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Abstract: Roots of crop species produce exudates with biologically active chemicals which are known to affect the growth of crops and weed species. An experiment was conducted at the Uganda National Crop Resources Research Institute, Namulonge during 2016 to identify compounds released in root exudates of potted *Cymbopogon nardus*, *Desmodium uncinatum*, upland rice (NERICA 1), *Mucuna pruriens* and *Zea mays* (LONGE 6H) at forty-five days after planting. This marked near the average stationary phase for test crop growth when secondary metabolite levels were high. Organic compounds in soils were extracted using solid-phase micro-extraction (SPME) and by solvent extraction. Samples were subjected to analysis using a 7890A Gas Chromatography system. Data files were transferred into a distinct folder and data was uploaded onto the XCMS online platform for pairwise comparison and other related statistical analyses in the National Institute of Science and Technology Library. The blank soil produced 15 terpenoids, two alcohols and one each of trihalomethanes, ethers, phenols, ketones, furans, alkanes and aldehydes. Cymbopogon exuded five terpenoids, one phenol and an alkane. Desmodium plant roots released three terpenoids, one alkane and a phenol. The rice crop produced eight terpenoids, two alkanes and a furan. Five terpenoids, one phenol and an alkane were released by the mucuna crop, while six terpenoids were found in maize soil. The profiled compounds from cymbopogon, desmodium, rice, mucuna and maize could be responsible for allelopathic properties expressed by the study crops in natural and agricultural ecosystems and could be used in synthesis and development of herbicides.

Key words: alkanes, cymbopogon, desmodium, exudates, phenols, rice, terpenoids.

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Introduction

A wide range of biochemicals are synthesized during the shikimate pathway or, in the case of essential oils, from the isoprenoid pathway and are not required for the metabolism of the allelopathic organisms (Hussain et al., 2011). Allelochemicals are a subset of secondary metabolites released from plant parts by leaching, root exudation, volatilization and residue decomposition in both natural and agricultural systems. Li et al. (2010) reported that allelochemicals can be classified into 10 categories according to their different structures and properties, namely: (i) water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes and ketones; (ii) simple lactones; (iii) long-chain fatty acids and polyacetylenes; (iv) quinines (benzoquinones, anthraquinones and complex quinines); (v) phenolics, flavonoids and tannins; (vi) cinnamic acid and its derivatives; (vii) coumarins; (viii) steroids and terpenoids; (sesquiterpene, lactones, diterpenes and triterpenoids). The compounds with negative allelopathic effects are important in plant defence against herbivory (Rice, 1984). Root exudates affect soil-borne pests, pathogens, microorganisms, soil nutrients, microbial ecology and allelopathic activities of other plant species (Inderjit, 2003).

Flores et al. (1999) have indicated that 5–21% of plant photosynthates are released via root exudation over time in the rhizosphere depending on the size of the root system, plant responses to biotic and abiotic factors. Root exudation can often be modified by abiotic and biotic factors as well as physical and biological soil factors. Chuihua et al. (2004) and Kong et al. (2006) reported that the allelopathic rice varieties P1312777 and Huagan-1 released momilactone B, 3-isopropyl-5-acetoxycycloxexane-2-one-1 and 5,7,4'-trihydroxy-3',5'-dimethoxyflavone into the soil. Kato-Noguchi (2011) identified two main inhibitory substances in rice exudates by spectral data as 3-hydroxy-β-ionone and 9-hydroxy-4-megastigmen-3-one. Kong et al. (2007) reported one aglycone, namely 5,7,4'-trihydroxy-3',5'-dimethoxyflavone, in the soil planted with allelopathic rice. Hooper (2010) reported *C*-glycosylflavones as the major compounds in the root exudates of *Desmodium uncinatum.* L DOPA allelochemical was reported to be exudated from the roots of *M. pruriens* (Soares et al., 2014; Vadivel and Pugalenthi, 2008). Kato-Noguchi (2010) identified three allelochemicals in the acetone extract from the mesocotyls and coleoptiles of maize seedlings as 5-chloro-6-methoxy-2-benzoxazolinone; 6-methoxy-2-benzoxazolinone and 2,4-dihydroxy-l,4-benzoxazin-3-one.

Upland rice-based ecosystems are characterised by mixed cropping and some plants are emerging key intercrops that provide alternative sources of revenue given the high demand for their products. In order to appraise the productivity of the rice ecosystems, it must be considered that allelopathic effects are produced by upland rice and some intercrops. Allelochemicals have been associated with weed

control (Tesio and Ferrero, 2010), growth of component crops (Soares et al., 2014), nutrient uptake (Cheng and Cheng, 2015) and, thus, they affect crop productivity. The bio-compounds in *C. nardus*, *D. uncinatum*, upland (NERICA 1), *M. pruriens* and *Z. mays* (LONGE 6H) that cause the allelopathic effects are, however, not well documented and there is little literature on the bio-compounds. The study aimed at identifying the organic compounds exudated via roots by the crops.

Materials and Methods

Profiling of the bioactive compounds

Potting in the screen house

A screen house study was conducted in 2016 based at the Uganda National Crops Resources Research Institute, Namulonge, Uganda. Five 4-day-old pre-germinated seeds each for rice, desmodium, mucuna and maize and five suckers of cymbopogon were potted separately. One pot without any plant was maintained as a control and about 120 ml of tap water was applied to each pot every two days for 45 days. One plant was uprooted and 100 g of soil collected from the middle to the bottom of each pot as representative samples at 50 days after planting. This time marked near the average stationary phase for test crop growth when secondary metabolites were presumed to be higher. The samples were oven-dried at 80 ^{o}C for 12 hours to constant weight for compound analysis.

Extraction and analysis of potential organic compounds from soil and plants

Organic compounds in soils were extracted using solid-phase micro-extraction (SPME) and by solvent extraction. Prior to the extraction, the SPME fibre was preconditioned for one hour at 250^{o}C under a stream of helium inside the gas chromatograph (GC) injection port liner. The SPME fibre used was gauge 24, 1 cm long, coated with divinyl benzene/ polydimethylsiloxane and with the film thickness of 65 µM. In a single manual injection, one gram of each soil sample was accurately weighed into a 10 ml airtight glass vial. The sample and blank extractions were placed into a thermostat heated block at 60 ^{o}C for 1 hour with the fibre exposed to the headspace for the entire duration. The fibre was retracted and introduced into the injection port of the GC in splitless mode. One gram of soil sample was accurately weighed into a 50 ml extraction tube and extracted with 10 ml of hexane by shaking at 250 revolutions per minute (rpm) in an orbital shaker for one hour. The two-milliliter extract was aliquoted into an Eppendorf tube and centrifuged at 5000 rpm for 10 minutes and 1 ml extract was aliquoted into a GC vial for injection. One hundred milligrams of freeze-dried samples were accurately weighed and each put into a 2 ml Eppendorf tube. 1800 µL of hexane and two mini

steel balls were also added to each of the Eppendorf tubes. The samples were vigorously ground in a genogrinder for 10 minutes. The extract was centrifuged at 5000 rpm for 5 minutes and 200 µL of the extract was diluted with 800 µL of hexane in a GC vial for injection.

Gas chromatography and mass spectrometry instrumental analysis conditions

Samples were subjected to analysis using a 7890A (Gas Chromatography) GC system (Agilent Technologies, USA) coupled to a 240 ion trap mass spectrometer (MS) detector (Agilent Technologies) using the Agilent 7693A automatic liquid sampler for solvent extracted samples. A VF5-MS (5% phenyl methylpolysiloxane), 30 m × 0.25 mm id, 0.25 µm film capillary column was used with the injector port set at 280 °C. Helium was used as carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed to rise from 50 °C to 180 °C at 4 °C/min followed by an increase to 250 °C at 3 °C/min. The ion trap mass spectrometer parameters were as follows: scan range 50–540 (m/z), ionization mode EI and transfer line temperature, manifold temperature and trap temperature of 250 °C, 100 °C and 150 °C, respectively. Chromatograms and spectra representing individual samples were analysed using the automated mass spectral deconvolution and identification system software (AMDIS, US). The identification of the individual compounds was performed by comparing each of the mass spectra with the database of the National Institute of Science and Technology (NIST) 11 (Gaithersburg, MD, USA) and Wiley 7N (John Wiley, NY, USA) and also by comparing the calculated Kovats linear retention indices using retention times of n-alkane series against the values obtained in the NIST web book for the same capillary column stationary phase.

Chemometric analysis of GC-MS raw data using XC-MS online platform

Data files in the common data formats corresponding to chromatograms from the various solvent extracted soil and SPME extracted volatiles in soil samples were obtained and transferred into a distinct folder. Data was uploaded onto the XCMS online platform for pair-wise comparison and other related statistical analyses. The analysis was performed using the default parameters under the GC/single quad (centwave) method as it matched the instrument operating conditions. The results obtained included: retention time corrected, total ion chromatograms, principal component analysis (PCA) plots and an annotated report. The generated report was used to identify the log-fold changes for various compounds earlier identified using AMDIS. Compounds with the log-fold changes $\geq$ 0.30 were considered generated from the test samples.

Results and Discussion

Total ion chromatograms for compounds in test soils overlaid on control

Graphical images of the total ion chromatograms (TIC) generated from the solid-phase micro-extraction (SPME) data files for compounds in soil treatment samples potted with *C. Nardus* (S_1), *D. Uncinatum* (S_2), upland NERICA 1 (S_3), *M. pruriens* (S_4) and *Z. mays* (S_5) overlaid against the blank soil sample (S_0) as control are presented (Figure 1). The TIC spectra were similar with minor variations in the signal intensities of the TIC overlaid. The observation may be attributed to the availability of similar compounds in the control (S_0) and treatment soils (S_1, S_2, S_3, S_4 and S_5).

Analysis using XCMS online

The PCA showed no clustering of the sample spectra. The generated PCA plot for the blank soil (S_0) was laid in quadrant 1. The PCA plots for *D. uncinatum* (S_2) and upland rice, NERICA 1 (S_3), were in the second quadrant (Figure 2). The PCA for *C. nardus* (S_1) was positioned in quadrant 3 and the PCA plots for *M. pruriens* (S_4) and *Z. mays* (S_5) were both located in the fourth quadrant. The PCA plots that clustered closer signified similarity of the metabolites in the test samples and vice versa. Plant samples with similar classes of compounds clustered in the same quadrant.

Total ion chromatograms

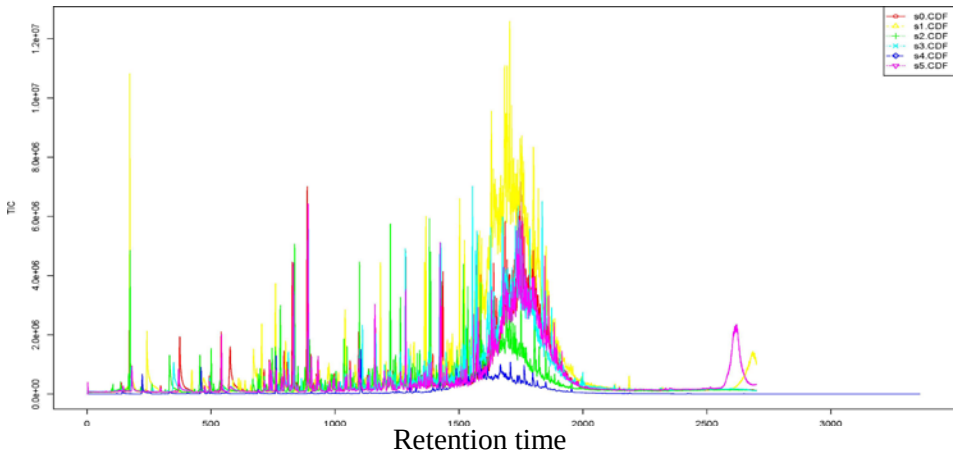

Figure 1. Generated total ion chromatograms (TICs) for organic compounds from cymbopogon (S_1), desmodium (S_2), NERICA 1 rice (S_3), mucuna (S_4) maize (S_5) and control (S_0) overlaid. CDF = Compatible document format.

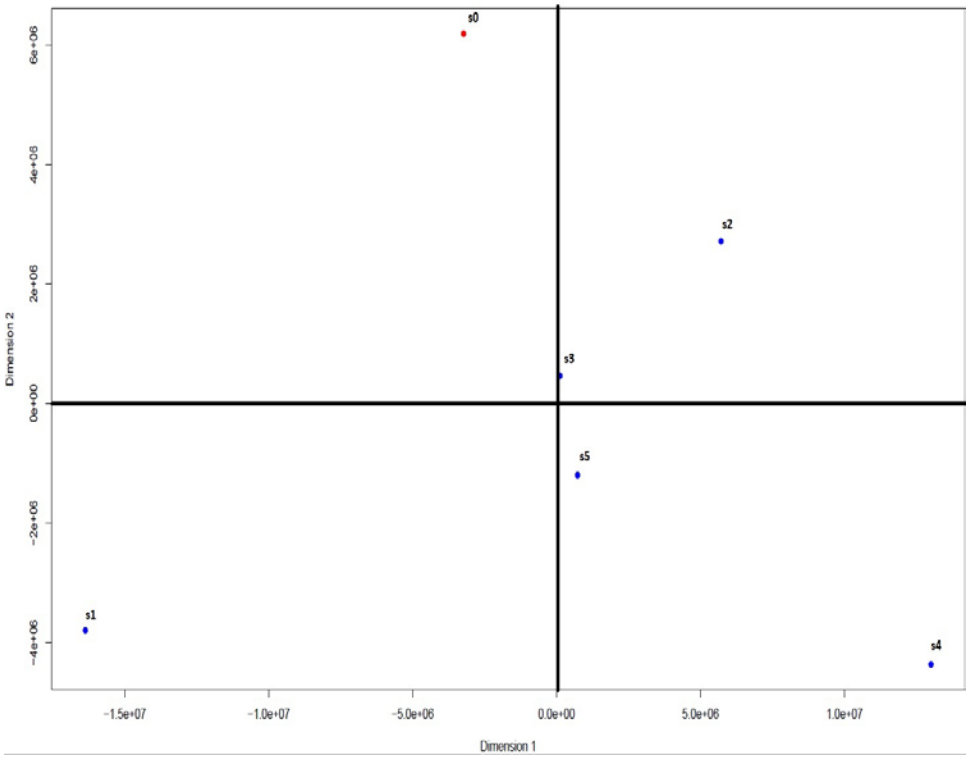

Figure 2. PCA plot showing the clustering of the various compounds in cymbopogon (S_1), desmodium (S_2), upland NERICA 1 (S_3), mucuna (S_4) maize (S_5) and control (S_0).

Bioactive compounds identified in the control and soil potted with test plants

Data on capillary column stationary phase retention time, relative match factors and the compounds identified in the control soil and soil potted with *C. nardus*, *D. uncinatum* and upland rice (NERICA 1) *M. pruriens* and *Z. mays* (LONGE 6H) are indicated in Tables 1–6. Twenty-four compounds were identified as the most probable compounds in the control (blank) soil (Table 1). This was dominated by fifteen terpenoids, namely: ethylbenzene, p-dimethylbenzene, vinyl benzene, o-methyl styrene, m-ethyl toluene, p-ethyl toluene, 1,3,5-trimethylbenzene, isopropyl benzene, 1,2,3-trimethylbenzene, o-dichlorobenzene, 1,2,4-trimethylbenzene, 1-isopropyl-2-methylbenzene, L-limonene, 3-phenylpropene and 1-3-diethylbenzene. Nine other compounds in the soil included

one trihalomethane called trichloromethane, n-butyl ether, oxime-methoxy-phenyl phenol, acetophenone ketone, 2-n-pentylfuran, 3,5-dimethyloctane alkane, n-octanal aldehyde and two alcohols identified as ethylhexanol and benzyl alcohol.

Table 1. Retention time, relative match factors and identified compounds in the blank soil.

Retention time (min)	Relative match factor	Identified compound
2.08	875	Trichloromethane
6.38	820	Ethylbenzene
6.73	861	p-dimethylbenzene
7.14	867	n-butyl ether
7.59	873	Vinyl benzene
8.19	810	Oxime-, methoxy-phenyl
9.98	795	o-methyl styrene
10.34	814	Acetophenone
10.72	915	m-ethyl toluene
10.84	854	p-ethyl toluene
11.13	897	1,3,5-trimethylbenzene
11.50	887	Isopropyl benzene
12.07	840	2-n-pentylfuran
12.22	883	1,2,3-trimethyl benzene
12.47	866	3,5-dimethyloctane
12.68	854	.n-octanal
13.13	919	o-dichlorobenzene
13.36	866	1,2,4-trimethylbenzene
13.52	888	1-Isopropyl-2-methylbenzene
13.69	868	L-limonene
13.80	822	2-ethylhexanol
13.90	789	3-phenylpropene
14.09	807	Benzyl alcohol
14.42	822	1,3-diethylbenzene

The twenty-four compounds identified in the control had the PCA plots (S_0) clustered solely in the first quadrant. This signified that the compounds differed from sample treatments (S_1-S_5). The observation is supported by the result that only five compounds profiled from the 5 test crops were also found in the control. It is presumed that the bio-compounds identified in S_0 had possibly been either released via root exudates by similar or different crops previously grown on the soil or were deposited from the decomposing plant materials. Rice (1984) and Uren (2000) have reported that root exudates contain different classes of primary and secondary compounds.

Seven compounds were profiled in the soil potted with *C. nardus*. One compound named 2-Ethylhexanol phenol, identified in the blank, was the only

major compound exudated with positive log-fold changes when overlaid with the control (Table 2). Three terpenoids named tert-Amylbenzene, pentamethylbenzine, 1,2-Di-tert-butylbenzene and 2,3-dimethylundecane alkane were released with high positive log-fold changes ($\geq$0.3), while naphthalene and 1-Sec-butyl-4-methylbenzene terpenoids were exudated with lower log-fold changes. The PCA plot for cymbopogon (S_1) solely lied in the third quadrant and far from S_0 in Figure 2, due to the lower similarity between the metabolites in cymbopogon and the control (S_0). There are no similar reported compounds in the available literature to the compounds profiled in root exudates of *C. nardus*.

Table 2. Retention time, relative match factors and log-fold changes for compounds in *Cymbopogon nardus.*

Retention time	Relative match factor	Positive log-fold change	Identified compound
13.80	859	0.70	2-Ethylhexanol
18.48	896	0.37	tert-Amylbenzene
18.98	892	0.26	1-Sec-butyl-4-methylbenzene
19.41	860	0.17	Naphthalene
19.70	876	0.53	Pentamethylbenzine
21.29	921	1.80	1,2-Di-tert-butylbenzene
18.83	867	0.39	2,3-Dimethylundecane

D. uncinatum produced five dominant organic compounds with positive log-fold changes ($\geq$ 0.3) that included one furan named 2-n-Pentylfuran which had been identified in the control (Table 3). Three terpenoids released via roots included tert-Amylbenzene; p-Ethyltoluene and 1-Sec-butyl-4-methylbenzene. An alkane named 2,3-Dimethylundecane was also exudated by *D. uncinatum* into the soil.

Table 3. Retention time, relative match factors and log-fold changes for compounds in *Desmodium uncinatum.*

Retention time (Min)	Relative match factor	Positive log-fold changes	Identified compound
10.84	876	0.36	p-Ethyltoluene
12.07	874	0.87	2-n-Pentylfuran
18.48	882	0.74	tert-Amylbenzene
18.83	867	0.42	2,3-Dimethylundecane
18.98	901	0.65	1-Sec-butyl-4-methylbenzene

Eleven compounds were released by upland rice (NERICA 1) in the root exudates that included one furan named 2-n-pentylfuran that had been isolated from the control treatment (Table 4). One alkane named 2,3-dimethylundecane and six terpenoids identified as 1,2-dimethyl-3-ethyl benzene, 1-methyl-2-(2-

propenyl)benzene, 1,3-di-tert-butylbenzene, 1-Sec-butyl-4methylbenzene, tert-amylbenzene and pentamethylbenzine were exudated dominantly. Three compounds released with lower log-fold changes included 2-ethyl-p-xylene and 1-methyl-3-propylbenzene terpenoids and an alkane identified as 2,3-dimethyloctane.

Table 4. Retention time, relative match factors and log-fold changes for compounds in upland NERICA 1.

Retention time	Relative match factor	Positive log-fold change	Identified compound
12.07	894	0.54	2-n-pentylfuran
12.47	883	0.23	3,5-dimethyloctane
14.56	856	0.11	1-methyl-3-propylbenzene
14.84	856	0.08	2-ethyl-p-xylene
15.53	876	0.90	2,3-dimethyldecane
15.87	843	0.37	1,2-dimethyl-3-ethyl benzene
18.14	857	0.78	1-methyl-2-(2-propenyl)benzene
18.48	877	0.76	Tert-amylbenzene
18.98	871	1.30	1-sec-butyl-4-methylbenzene
19.70	867	0.83	Pentamethylbenzine
21.29	869	0.34	1,3-di-tert-butylbenzene

The five and eleven compounds profiled in the root exudates of desmodium and rice respectively were characteristically represented by the closely positioned PCA plots for both crops in the second quadrant, signifying a common association between their secondary metabolites (Figure 2). Two similar terpenoids, namely 1-Sec-butyl-4 methylbenzene and Tert-Amylbenzene, one furan named 2-n-Pentylfuran and 2,3-Dimethylundecane alkane were commonly produced by the two crops. Hooper (2010) reported C-glycosylflavones as the major compounds in the root exudates of *D. uncinatum.* Several researchers have reported different compounds released by various cultivars of rice. Kong et al. (2007) reported momilactone B, 5,4-dihydroxy-3,5-dimethoxy-7-O-b-glucopyranosylflavone, 3-isopropyl-5-acetoxycyclohexene-2-one-1 and flavone, O-glycoside as released by rice roots. Kato-Noguchi et al. (2008) reported that rice secreted momilactones A and B into its rhizosphere. Kato-Noguchi (2011) identified 3-hydroxy-β-ionone and 9-hydroxy-4-megastigmen-3-one metabolites in rice root exudate. The profiled secondary metabolites were different from available literature and this may be attributed to differing genetic crop influences. Kim and Shin (1996) have reported that allelochemicals are influenced more by genetics than by the environment.

Mucuna pruriens released six organic compounds with high log-fold changes in its root exudates which included five terpenoids, namely: naphthalene, 1,2-dimethyl-4-ethylbenzene, 1,3-ditertiarybutylbenzene, 1-ethyl-3-methyl-benzene and 1,3-dichloro-benzene (Table 5). An alkane identified as n-Tetradecane was also profiled. Six compounds were exudated by *Z. mays.* A furan 2-n-pentylfuran

and three terpenoids, namely: m-ethyltoluene, 1,2,4 Trimethylbenzene and 2-ethyl-p-xylene which had been identified in the control soil (Table 6) were also profiled in the soil potted with *M. pruriens*. Two terpenoids, namely: 1-methyl-2-(2-propenyl) benzene and o-dichlorobenzene, were profiled with lower log-fold changes from the mucuna soil. The two terpenoids, namely: m-ethyltoluene and 2-ethyl-p-xylene, were dominant ($\geq$ 0.3) in the soil potted with LONGE 6H maize.

The PCA plots for mucuna (S_4) and maize (S_5) were both in the fourth quarter and closely clustered. This may be attributed to the dominance of a high number (5 and 6) of terpenoids released by mucuna and maize crops respectively via root exudates. The mucuna crop, however, clustered distantly from the control treatment relative to the maize crop. This was possible because mucuna and the control produced no similar compounds, while maize exudated four common compounds with the control.

Table 5. Retention time, relative match factors and log-fold changes for compounds in *Mucuna pruriens.*

Retention time	Relative match factor	Positive log-fold change	Identified compound
8.00	895	0.38	Naphthalene
12.45	899	052	1,2-Dimethyl-4-ethyl benzene
15.06	875	0.46	n-Tetradecane
16.71	872	0.31	1,3-Ditertiarybutylbenzene
8.27	869	0.43	1-ethyl-3-methyl-benzene
8.97	877	0.48	1,3-dichloro-benzene

Table 6. Retention time, relative match factors and log-fold changes for compounds in *Zea mays* (LONGE 6H).

Retention time	Relative match factor	Positive log-fold change	Identified compound
10.72	866	0.30	m-Ethyltoluene
12.07	894	0.17	2-n-Pentylfuran
18.14	891	0.07	1-Methyl-2-(2-propenyl)benzene
13.13	897	0.11	o-Dichlorobenzene
13.36	854	0.16	1,2,4-Trimethylbenzene
14.84	842	0.31	2-Ethyl-p-xylene

The PCA plots for rice (S_3) and maize (S_5) were close to each other and to the PCA for the control (S_0). This may be attributed to the common presence of 2-n-pentylfuran in the three treatments. Despite cymbopogon and rice clustering in different quadrants, the PCAs for both crops were close possibly due to the commonly exudated tert-amylbenzene, pentamethylbenzine and 1-sec-butyl-4-methylbenzene terpenoids and 2,3-dimethylundecane alkane. There are no similar metabolites reported in the root exudates of *M. pruriens* to the ones profiled under

the current study. Nishihara et al. (2004) and Soares et al. (2014) reported L-DOPA to be dominantly exudated from the roots of *M. pruriens*. Kato-Noguchi (2010) identified 3 different allelochemicals, namely: 5-chloro-6-methoxy-2-benzoxazolinone, 6-methoxy-2-benzoxazolinone and 2,4-dihydroxy-l,4-benzoxazin-3-one from the mesocotyls and coleoptiles of rice seedlings.

Generally, the profiled compounds had not been previously identified and reported in the literature. This may be attributed to variation in the genetic characteristics of the test plants and environmental conditions. Jensen et al. (2001) have revealed that allelopathy is quantitatively inherited and identified four main quantitative trait loci (QTL) on three chromosomes of 142 cultivars of upland rice that collectively explained 35% of the allelopathic activity in the population. The potential effects of some bioactive compounds from the test crops have been reported by researchers. Kato-Noguchi (2011) and Poonpaiboonpipat et al. (2013) reported allelochemicals in rice and cymbopogon root exudates to inhibit weed growth. Citral of cymbopogon was reported to cause disruption of microtubules in wheat and *Arabidopsis thaliana* L. roots by Chaimovitsh et al. (2012). Ayeni and Kayode (2014) recorded inhibited seed germination by compounds in root extracts and tassel of maize. Reduced growth of component and subsequent field crops was observed by Pickett et al. (2010) and Soares et al. (2014), in desmodium and mucuna crops, respectively. The effects were attributed to allelochemicals released via root exudates. Cheng and Cheng (2015) reported allelochemicals to inhibit the absorption and transport of ions at the cell plasma membrane in various crops.

Conclusion

Bioactive compounds were profiled in soils potted with *C. nardus*, *D. uncinatum*, *M. pruriens*, upland rice (NERICA 1) and *Z. mays* (LONGE 6H). The secondary metabolites included terpenoids, phenols and alkanes. Terpenoids were the principal compounds. The results demonstrate that *Cymbopogon nardus*, upland rice (NERICA 1) *Desmodium uncinatum*, *Mucuna pruriens* and *Z. mays* (LONGE 6H) produced bio-compounds in their root exudates. Some of the compounds are reported to exhibit allelopathic properties and could be significant in the establishment and development of cultivated and natural ecosystems; declines in crop yield due to reduced uptake of nutrients, crop regeneration failure and replant problems. The study crops have been reported to exhibit negative and positive allelopathic influences. Further studies are recommended on the allelopathic potential of the specific compounds identified under specialised crop treatments to allow efficient generation of appropriate allelopathic cultivars. Such cultivars could become important tools in the development of advanced integrated weed management.

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PROFILI JEDINJENJA U EKSUDATIMA KORENA PIRINČA, LIMUN TRAVE, DEZMONIUMA, STIZOLOBIUMA I KUKURUZA

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R e z i m e

Korenovi ratarskih biljaka proizvode eksudate sa biološki aktivnim hemikalijama za koje je poznato da utiču na rast useva i korovskih vrsta. Ogled je sproveden u Nacionalnom državnom institutu za istraživanje ratarskih biljaka u Ugandi, Namulonge tokom 2016. godine kako bi se identifikovala jedinjenja koja se oslobađaju u eksudatima korena biljaka *Cymbopogon nardus*, *Desmodium uncinatum*, planinskog pirinča (NERICA 1), *Mucuna pruriens* i *Zea mays* (LONGE 6H) gajenih u sudovima četrdest i petog dana posle setve. Ovo je označavalo period blizu prosečne stacionarne faze za rast ispitivanih useva kada su nivoi sekundarnih metabolita visoki. Organska jedinjenja u zemljištima su ekstrahovana korišćenjem mikroekstrakcije u čvrstoj fazi (engl. *solid-phase micro-extraction* – SPME) i ekstrakcijom rastvarača. Uzorci su analizirani korišćenjem 7890A gasnog hromatografskog sistema. Datoteke podataka prebačene su u poseban dokument i podaci su preneseni na onlajn platformu XCMS radi poređenja parova i druge statističke analize u biblioteci Nacionalnog instituta za nauku i tehnologiju. Na kontrolnoj varijanti (samo zemljište) identifikovano je 15 terpenoida, dva alkohola i svaki od njih trihalometane, etere, fenole, ketone, furane, alkane i aldehide. Limun trava je izlučila pet terpenoida, jedan fenol i jedan alkan. Korenovi biljke dezmodium izlučili su tri terpenoida, jedan alkan i fenol. Usev pirinča proizveo je osam terpenoida, dva alkana i jedan furan. Pet terpenoida, jedan fenol i jedan alkan oslobođeni su iz varijante sa stizolobiumom, dok je šest terpenoida pronađeno u varijanti sa kukuruzom. Profilisana jedinjenja iz limun trave, dezmodiuma, pirinča, stizolobiuma i kukuruza mogle bi biti odgovorne za alelopatske osobine koje su se ispoljile kod istraživanih useva u prirodnim i poljoprivrednim ekosistemima, te bi se mogle koristiti u sintezi i stvaranju herbicida.

Ključne reči: alkani, limun trava, dezmodium, eksudati, fenoli, pirinač, terpenoidi.

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GENETIC POLYMORPHISM OF *β*-LACTOGLOBULIN GENE IN INDIGENOUS NIGERIAN GOAT BREEDS

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Abstract: Polymorphism at the β-Lactoglobulin (β-LG) gene of three Nigerian goat breeds, namely: the West African Dwarf, Sahel and Red Sokoto goats, was investigated using the Polymerase Chain Reaction-Random Fragment Length Polymorphism (PCR-RFLP) method. The restriction endonucleases used in the study were Rsal and Mspl, respectively. The results revealed the existence of only one polymorphic variant (allele A) with a gene frequency of 1.0 in all the three goat breeds studied. The amplified products were observed at 120 bp and the restriction digestion with Rsal revealed just one genotype at the β-LG locus. It was concluded that there was the absence of polymorphism at the β-LG locus of the goats investigated.

Key words: polymorphism, goats, gene locus, PCR-RFLP, *β*-Lactoglobulin.

Introduction

Domestic goats are kept extensively at a global scale majorly in the developing world and serve as a good source of milk, meat, fibre and pelts (MacHugh and Bradley, 2001; Qureshi et al., 2014). Variability in economic traits has been recorded among different goat breeds and within breeds as well. Researchers have established that gene polymorphism can affect the yield of milk and coding for whey proteins (Kumar et al., 2006; El-Hanafy et al., 2010). Genetic polymorphism is the incidence in a population of several alleles at one locus, each with considerable frequency, where the least occurrence is usually taken as 1% (Philip, 2011). It comes about as a result of chance processes or may have been induced by external agents such as viruses, chemicals or radiation. The main factors that can lead to phenotypic variation in an organism are basically its genotype and environmental factors acting on it.

Variations in the DNA sequences can be studied by protein polymorphism. *β*-Lactoglobulin (*β*-LG) is the dominant non-casein whey protein present in the milk

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of goats. It has widely been accepted to be absent in humans, although there are indications of minor presence (Hambraeus and Lonnerdal, 2003). Studies have revealed *β*-LG-pectin complexes as molecular nano-vehicles for delivering hydrophobic nutraceuticals such as fatty acids and vitamin D (Kontopidis et al., 2004; Zimet and Livney, 2009; Ron et al., 2010; Cui et al., 2012). Studies exploring *β*-LG polymorphism in goats have found two allelic variants: A and B (Kumar et al., 2006; El-Hanafy et al., 2010). El-Hanafy et al. (2010), in their study of goats in Egypt, observed three genotypes (AA, AB, BB) of *β*-LG gene with genotype frequency of 0.1, 0.8, 0.1; 0.85, 0.1, 0.05 and 0.41, 0.51, 0.08 in Barki, Damascus and Damascus x Barki crossbred, respectively. El-Hanafy et al. (2014) reported the presence of three genotypes (AA, AB, BB) in three Saudi goats with genotype frequencies of 0.08, 0.4, 0.52 (Ardi), 0.23, 0.41, 0.36 (Habsi) and, 0.09, 0.34, 0.57 (Harri) goats, respectively. The authors reported allele frequency for the A and B polymorphic forms of the gene in the goats as 0.28 and 0.72 (Ardi), 0.43 and 0.57 (Habsi) and, 0.26 and 0.74 (Harri), respectively. The *β*-LG locus in Spanish and French goats was characterized at the DNA level revealing two new genetic variants (Pena et al., 2000). Chianese et al. (2000) also observed differences in the *β*-LG content of Italian Girgentana goats ranging from 43 to 63% of the major whey protein in the milk. A polymorphism in the promoter region of each Italian Girgentana goat with reduced β-LG content was identified (Graziano et al., 2003) even though it has not been correlated with β-LG content of the milk. The forms of *β*-LG gene and frequency at the locus have not been studied in indigenous Nigerian goats. Therefore, the aim of this research was to investigate the genetic polymorphism of the *β*-lactoglobulin locus in indigenous Nigerian goat breeds using PCR-RFLP methods.

Materials and Methods

Blood samples used for DNA isolation were collected from 60 goats belonging to three indigenous Nigerian goat breeds: Red Sokoto (20), Sahel (20) and West African Dwarf (20). The blood samples were collected from goats kept by the National Animal Production Research Institute (NAPRI), Shika, Zaria, Kaduna State and from Ibadan metropolis. Total DNA was isolated from whole blood samples using a ZymoBeadTM Genomic DNA Kit using the protocol recommended by the manufacturer (Zymo Research Corporation). The Zymo Research (ZR) kit was used in the present study for the isolation and extraction procedure of genomic DNA from the blood samples collected from the goats because the procedure has been reported to yield more DNA than was observed when using other methods in reef corals (Santos et al., 2012); it was also reported to be more time-saving and cost-effective than other extraction methods for

forensic samples (Yunjie and Oluseyi, 2013). Gel monitoring was used to determine DNA quality.

PCR and RFLP procedure

β-Lactoglobulin genotypes were identified as described by Feligini et al. (1998). The β-lactoglobulin genotypes were identified in two steps: in the first step, the 120 bp (base pair) fragment of the goat β-lactoglobulin gene was amplified using forward primer 5-CAACTCAAGGTCCCTCTCCA-3 and reverse primer 5-CTTCAGCTCC TCCAGGTACA-3. PCR amplifications were performed in reaction mixtures of 25μL containing 12.5μL of 2×PCR master mix (ZymoBiomics™ PCR PreMix), 0.5μM of each primer, and 25-75ng genomic DNA. Amplification was performed in a Biologix Thermal Cycler (TC1000-G), programmed for an initial denaturation at 95°C for 10 minutes, followed by 35 cycles each with denaturing at 93°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

In the second step, the 105 bp fragment of the goat β-lactoglobulin gene was amplified using forward primer 5-TCAGGACCCCGGAGGTGGACAAC-3 and reverse primer 5-CCTCCAGCTGGGTCGGGTTGAAG-3. The cycling program began with an initial denaturation step (1 min at 94°C), followed by 30 cycles consisting of 15 seconds at 94°C, 1 minute at 60°C, 10 seconds at 72°C, and a final elongation for 10 minutes at 72°C. The same PCR reaction mixtures used in the first step were used for amplification. In both cases, PCR products (12 μL) were digested with 8 U of *Ras*a and 10 U of Mspl restriction enzyme in a 20μL total reaction volume for 2 hours at 37°C. Mspl is a restriction endonuclease obtained from the organism *Moxarella spp.* The restriction fragments were directly analyzed by electrophoresis in 3% agarose gel in 1×TAE buffer stained with ethidium bromide and visualized under Ultra Violet (UV) light. The genotypes of the analyzed individuals at the β-lactoglobulin locus were recognized using the restriction fragments observed in the gel.

Statistical analysis

Direct counting was used to estimate phenotypic and allele frequencies of β-lactoglobulin genetic variants. The chi-square test (χ2) was used to check for whether the populations were in Hardy-Weinberg equilibrium. All calculations and the χ2 analyses were carried out using GenAIEx software v. 6.502 (Peakall and Smouse, 2012).

Results and Discussion

Isolation of genomic DNA of *β*-lactoglobulin gene of indigenous Nigerian goat breeds

Figure 1 shows the agarose gel electrophoresis results of DNA of *β*-lactoglobulin gene extracted from Sahel, Red Sokoto and West African Dwarf goats. The isolated DNA was of high quality, high molecular weight and appeared as single bands without sheared fragments. There are many molecular techniques available for the extraction and purification of genomic DNA from blood and other animal tissues. Different biotechnological laboratories derive different techniques, depending on the facilities available to obtain results.

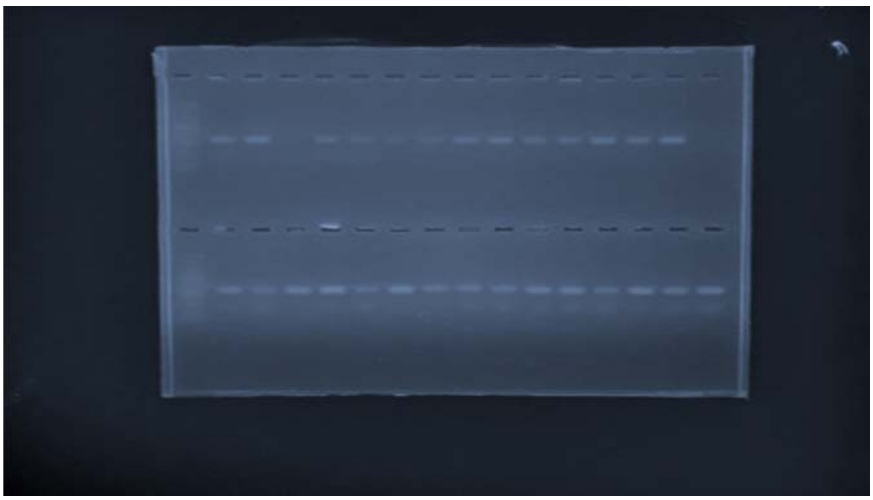

Figure 1. Agarose gel electrophoresis of genomic DNA extracted from Nigerian indigenous goat breeds.

The high quality, high molecular weight and single banded and sheared-less DNA fragments obtained in the present study agree with the reports of Santos et al. (2012). It is, however, in disagreement with the findings of Yunjie and Oluseyi (2013), who reported that the highest yield of DNA was obtained when using the Qiagen kit followed by the Bioneer kit and Zymo kit in that order. The Zymo research kit was adopted in this study because its protocol was found to be simple and unambiguous. This is supported by Yunjie and Oluseyi (2013); the authors recommended the use of the Zymo genomic DNA kit in laboratories where the speed of sample processing is paramount.

PCR amplification of the β-lactoglobulin gene of indigenous Nigerian goat breeds

The agarose gel electrophoresis results of the PCR amplified β-lactoglobulin gene are presented in Figures 2 and 3, respectively. In all the samples tested, the amplified product size was approximately 120 and 105 bp, respectively, with no variation in size between the animals studied.

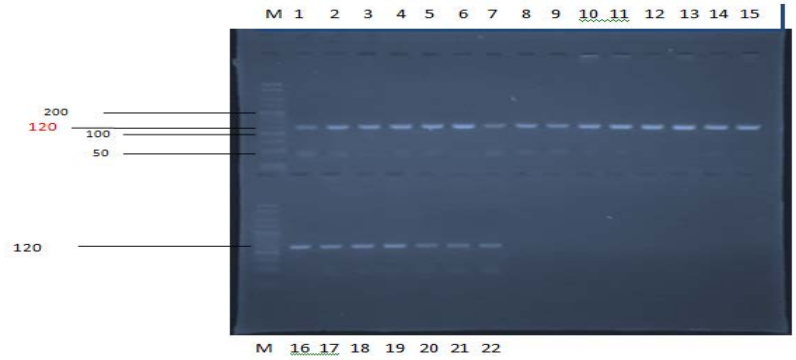

Figure 2. Agarose gel electrophoresis of PCR amplified product of exon II from position 1563 of intron I to position 1779 of intron II of the β-lactoglobulin gene of indigenous Nigerian goat breeds Lane M = 25 bp DNA ladder; Lane 1-22 = PCR amplicons.

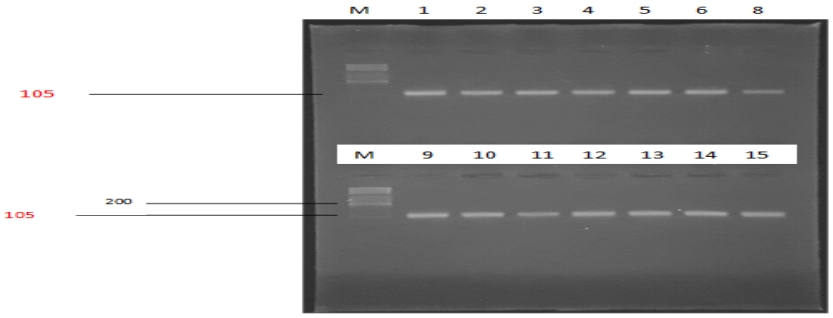

Figure 3. Agarose gel electrophoresis of PCR amplified products of exon V from position 4551-4655 of the β- lactoglobulin gene of indigenous Nigerian goat breeds PCR-RFLP analysis of the β-lactoglobulin gene of indigenous Nigerian goat breeds.

The 120 bp fragments of the exon II from position 1563 of intron I to position 1779 of intron II were observed to be similar for all the animals to the 105 bp

fragments of exon V from position 4551-4655 of the *β*-lactoglobulin gene of the indigenous breed of goats amplified by PCR using oligonucleotide primers for both steps. This result confirms the repeatability of the method of identification of the *β*-lactoglobulin genotype described by Feligini et al. (1998) indicating that the *β*-lactoglobulin gene locus is conserved in the goat breeds studied.

The PCR-RFLP analysis of the *β*-lactoglobulin gene extracted from indigenous Nigerian goat breeds is presented in Figures 4 and 5, respectively.

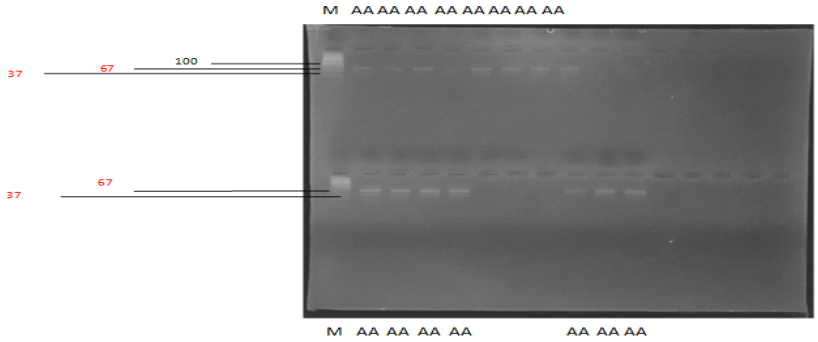

Figure 4. Agarose gel electrophoresis of 120 bp *β*- lactoglobulin genotyping by PCR-RFLP with Rsal enzyme of native Nigerian goats Lane M = DNA ladder; Lane 1-12 = AA genotype; Lane 15-18 = AA genotype.

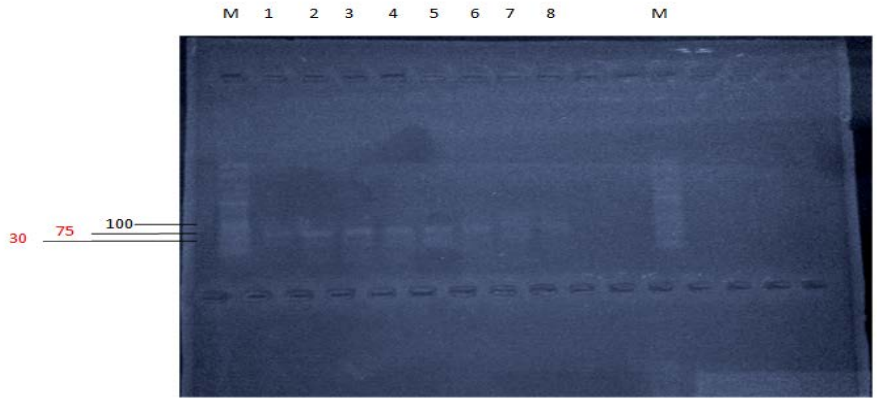

Figure 5. Agarose gel electrophoresis of 105 bp *β*-lactoglobulin genotyping by PCR-RFLP with Mspl enzyme of Nigerian indigenous breed of goats.

The first step which involved the use of Rsal restriction enzyme revealed the presence of only A genetic variant at the *β*-lactoglobulin gene locus in the amplified 120 bp PCR products (Figure 4). The second step using Mspl showed

that it digested the PCR fragments into two fragments of size 75 and 30 bp (Figure 5). No other RFLP pattern was observed from the agarose gel. The presence of only one allele A at the β-lactoglobulin gene locus at the amplified 120 bp PCR product is in agreement with earlier reports (Yahyaoui et al., 2000). The authors also recorded the presence of only one genetic variant at the β-lactoglobulin gene locus of a Lithuanian goat (Canaria). The Rsal-RFLPs allelic pattern observed contradicts earlier reports (El-Hanafy et al., 2010). The authors recorded the presence of two genetic variants; A and B using Barki, Damascus and, Damascus x Barki crossbred in Egypt.

These differences observed in the results could be because different restriction enzymes were used in the studies. Elyasi et al. (2010), however, reported the presence of variants A, B and AB in Iranian goats using the Rsal restriction enzyme. Other authors who have reported on the presence of the AB variant in goats are El Hanafy et al. (2010, 2014). The presence of a single allele at the β-lactoglobulin gene locus can be attributed to mating (inbreeding). Inbreeding reduces heterozygosity at the same time as it is escalating the percentage of homozygotes relative to random expectations (Janna et al., 2015). Genetic variation is usually revealed by increasing heterozygosity and allelic diversity. Woodworth et al. (2002) reported that genetic diversity can be reduced as a result of genome-wide activities like inbreeding and genetic drift, and even the effect of practices like the artificial selection on individual genes. The 75 and 30 bp fragments observed with the use of the Mspl restriction enzyme to check for the β-lactoglobulin gene C variant conformed to the reports of Elmaci et al. (2007). Elmaci et al. (2007) have reported that the β-lactoglobulin gene C allele is characterized by only a 105 bp fragment. No other Mspl-RFLP allelic pattern was observed from the agarose gel, thus indicating the absence of C variant at the β-lactoglobulin gene locus of the Nigerian indigenous goat breeds sampled. The rare variant (β-lactoglobulin gene C) has, however, been detected in few goat breeds such as the Jamunapari and Jakhrana Indian goats (Jain et al., 2012) where it was detected in exons 3, 6 and 7 of the DNA. A, B and C alleles are generally found more in indigenous goat breeds (Barłowska et al., 2007; Torres-Vázquez et al., 2008) than in typical dairy breeds. The appearance of only A allele of β-LG contradicts the result above and those of Kumar et al. (2006) and El Hanafy et al. (2010, 2014).

Allelic and genotypic frequencies of β-lactoglobulin polymorphism of indigenous Nigerian goat breeds

Table 1 shows the allelic pattern of the β-lactoglobulin gene digested with the Rsal restriction enzyme in the indigenous Nigerian breed of goats while Table 2 shows the gene and genotype frequencies of β-lactoglobulin/Rsal polymorphism in

the indigenous Nigerian breed of goats. Only the A allele was found in the entire goat sample with fragment sizes of 66, 37 and 17, respectively (Table 1). The gene frequencies for the A and B alleles in Sahelian goats were 1.00 and 0.00, respectively. The same results were also observed in the Red Sokoto and West African Dwarf goats. The genotypic frequencies of *β*-lactoglobulin polymorphism for AA, AB and BB were observed as 1.00, 0.00 and 0.00, respectively for all goat breeds examined. These genotype frequencies were found not to be in Hardy-Weinberg equilibrium as the breeds were monomorphic at the *β*-lactoglobulin gene locus (Table 2). The monomorphic allelic pattern and genotype frequencies obtained in this study are in disagreement with the findings of El-Hanafy et al. (2010). The monomorphic allelic pattern, however, conforms with the report of Baltrėnaitė and Miceikienė (2007) who have reported that, in goat species, *β*-lactoglobulin gene protein is considered to be monomorphic due to the high frequency of *β*-lactoglobulin A genetic variant (ranging from 0.73 to 1.00). This was observed in Spanish, Hungarian and Lithuanian native goats. Considering the preponderance of the *β*-lactoglobulin A allele over the B allele, the A allele may well be taken as the inherited variant of the gene in Nigerian goats.

Table 1. The allelic pattern of the *β*-lactoglobulin gene digested with Rsal restriction enzyme in indigenous Nigerian breeds of goats.

S/No.	Allele type	Fragment size (bp)	Number of animals (n=60)
1	AA	66, 37, 17	60
2	BB	103, 17	0
3	AB	Both A and B fragments	0

Table 2. Genotype and allelic frequencies of the *β*-lactoglobulin gene locus in three indigenous Nigerian goat breeds.

		β-lactoglobulin genotype			Gene frequency		
Breed	Number	AA	AB	BB	A	B	$(\chi^2)^b$
Sahelian	20	20 (1.00)	0(0.00)	0(0.00)	1.00	0.00	Monomorphic
Red Sokoto	20	20 (1.00)	0(0.00)	0(0.00)	1.00	0.00	Monomorphic
WAD	20	20 (1.00)	0(0.00)	0(0.00)	1.00	0.00	Monomorphic
Total	60	60 (1.00)	0(0.00)	0(0.00)	1.00	0.00	Monomorphic

WAD = West African Dwarf, AA = *β*-LG AA, AB = *β*-LG AB, BB = *β*-LG BB, b = test of Hardy-Weinberg equilibrium.

The populations were found not to be in Hardy-Weinberg equilibrium which might be due to non-random mating (inbreeding) with its attendant increase in homozygosity of the gene leading to heterozygote deficiency. Elyasi et al. (2010) also reported similar findings in their study of polymorphism of *β*-lactoglobulin in Iranian goats using PCR-RFLP.

Conclusion

This research provides evidence that the *β*-LG locus of West African Dwarf, Red Sokoto and Sahelian goats is monomorphic. The singular nature of the *β*-LG locus points to a decrease in genetic diversity. This may have a negative effect on the population structure of the indigenous goats due to inbreeding depression. Its attendant consequences, especially on dairying ability of the studied goats, may lead to reduced productivity. Sometimes, results from different investigations are not comparable with each other for many reasons such as population size, breed, kind of primers used and restriction enzymes used.

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GENETIČKI POLIMORFIZAM β-LAKTOGLOBULINA KOD DOMAĆIH NIGERIJSKIH RASA KOZA

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R e z i m e

Ispitivan je polimorfizam β-Laktoglobulina (β-LG) kod tri nigerijske rase koza: zapadnoafričke patuljaste, sahel i crvene sokoto koze, korišćenjem metoda reakcije lančanog umnožavanja-polimorfizma dužine slučajnih delova (engl. *Random Fragment Length Polymorphism* – PCR-RFLP). Restrikcione endonukleaze korišćene u istraživanju uključivale su Rsal odnosno Mspl. Rezultati su otkrili postojanje samo jedne polimorfne varijante (alel A) sa frekvencijom gena od 1,0 kod sve tri rase koza koje su proučavane. Pojačani proizvodi uočeni su kod 120 bp, dok je restrikciona digestija sa endonukleazom Rsal otkrila samo jedan genotip kod lokusa β-LG. Zaključeno je da postoji odsustvo polimorfizma kod lokusa β-LG koza koje su ispitivane.

Ključne reči: polimorfizam, koze, lokus gena, PCR-RFLP, *β*-Laktoglobulin.

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BIOPROSPECTING NEGLECTED BOTANICALS AS PHYTO-FERTILIZERS FOR UNDERUTILIZED FOOD CROPS

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Abstract: Botanicals are part of the bioresources for sustainable growth of food crops, however, some botanicals have been neglected. This study investigates the potential effects of neglected botanicals on selected underutilized food crops (millet, sorghum and sesame) in Nigeria. Blotter technique and soil inoculation methods were used to evaluate the best botanicals that could be employed to enhance seed germination (SG) and seedling growth of underutilized food crops. With the exception of *Newbouldia laevis*, other botanicals enhanced seed germination of all tested crops. *Ficus asperifolia* and *Parquetina nigrescens* significantly ($p<0.05$) enhanced the radicle length (RL) while the plumule length (PL) remained unaffected. Specifically, observation shows that *Newbouldia laevis* completely inhibited sesame seed germination and seedling growth while *Parquetina nigrescens* enhanced millet height and sesame number of leaves. Generally, the effect of the botanicals on each crop was specific. Thus, these neglected botanicals stand the chance to enhance and sustain seedlings of underutilized food crops in Nigeria. Further study would be carried out to uncover the chemical components in the tested botanicals.

Key words: fertilizer, growth, botanical, neglected and underutilized crops, plants.

Introduction

As the world population increases, there is a need to re-strategize on how to feed nations (FAO, 2017), especially on the use of food crops. Apart from the fact that economically important food crops which include, but are not limited to, rice, maize, wheat, vegetables and leguminous crops (FAO, 2018; GIEWS, 2018) have overshadowed underutilized food crop species, they are also not enough to feed the world population (Li and Siddique, 2018). Of the 30,000 underutilized edible crop species, a mere 30 are used to feed the world. These underutilized crops can help to increase nutrient contents and diversification in food production. In addition to

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diversifying nutritional intake, underutilized crops provide not only economic and environmental benefits, but farmers can also grow them on their own (FAO, 2018; Li and Siddique, 2018).

Since these underutilized food crops are important to aid the global availability and nutri-beneficial foods, thus it is necessary to enhance their growth through the use of fertilizers. In addition to health implications (Southland Organic, 2012; Buckler, 2018), an excessive rate of chemical fertilizers also causes soil nutrient deterioration, soil structure deformation, heavy metal accumulation as well as the low yield of crops (Savci, 2012; Rahman and Zhang, 2018). On this note, environmental and health-friendly bio/phyto-fertilizers are the best alternatives. Among the phyto-fertilizers, botanicals have not been explored on seed germination and growth of underutilized crops in Nigeria.

Botanicals are substances extracted from plants which are used in pharmaceutical and cosmetic products, as food ingredients, and also as plant protection products (Okrikata and Oruonye, 2012; Seiber et al., 2014; Damalas and Koutroubas, 2018). Extracts from botanicals especially trees and crop residues have the potentials to influence crop growth and yield (El Atta and Bashir, 1999; Ahmed and Nimer, 2002; Farooq et al., 2008). *Sesbania sesban*, *Leucaena leucocephala*, *Eucalyptus microtheca* (Hussein and Abbaro, 1997) and *Moringa oleifera* are botanicals that have been established specifically to accelerate and strengthen the growth of young plants and crops. They also have the potentials to improve resistance to pests and diseases, increase leaf area as well as the number of roots and crop yield (Fuglie, 1999).

In Africa, especially Nigeria, many botanicals have been neglected and their potentials to enhance seed germination and seedling growth of crops have been relegated to the background (Murray, 2013). Apart from their pesticidal properties, there is little or no information regarding their phyto-fertilizer activities (Ertani et al., 2013; Ziosi et al., 2012). To ascertain the on-farm effect, the crude extracts of *Ficus asperifolia* (FA), *Parquetina nigrescens* (PN) and *Newbouldia laevis* (NL) were prepared exactly the way they are prepared by rural farmers and were evaluated on seed germination and seedling growth of millet, sorghum and sesame.

Material and Methods

Preparation of botanicals: Fresh leaves of FA, PN and NL were plucked from a local village around the Ibadan axis (Latitude – 7.30, Longitude – 3.38, Elevation – 176m) in Nigeria, and were properly washed in clean water. The samples were air-dried to obtain 0% moisture and blended in a clean electric blender (Qlink blender, Model number: QBL-20L 40, China). Exactly 10g of each botanical were added into 100ml of clean water and each sample was filtered twice through cheesecloth, collected in a flask and stored at 4^{o}C to be used later. Pearl millet

(*Pennisetum glaucum* – ExBronu), sorghum (*Sorghum bicolor* – NG/SA/JAN/09/088*)* and sesame (*Sesamum indicum* – white/raw) seeds were used for the experiment.

Blotter technique: Prior to inoculation, the Petri dishes were underlaid with sterilized moistened filter paper. This was followed by botanical inoculation (seeds were soaked in each crude extract for 3 minutes) and air-dried at room temperature (25 ± 2^{o}C). Briefly, 4 inoculated seeds of millet, sorghum and sesame were separately and appropriately placed at an approximate equidistant position to each other in 9-cm-diameter Petri dishes and incubated for 7 days at a temperature of 25 ± 2^{o}C (ISTA, 2003). At day 7, the germinated seeds were counted and percentage seed germination was determined using the formula: %G = n/Nx100. The radicle and the plumule lengths were measured with a thread and the precision was determined by the ruler. This experiment was done in three replicates and results were compared with the control (water).

Soil inoculation: Each pot (30 cm in diameter) contained 2.5 kg of sterilized soil; the soil was separately inoculated with 5 ml of each crude extract and homogeneously mixed with a sterile rod to maintain uniformity. After 24 hours, 4 seeds of millet, sorghum and sesame were sowed separately at the depth of 2 cm into the treated soil while the untreated seeds served as the control (seeds without botanicals). Using a completely randomized design, pots were arranged in three replicates. Watering was done throughout the experiment based on the requirement. At day 7, the interactive effect of each crude extract per crop was established. Percentage seed germination was determined using the formula: %G = n/Nx100. Data obtained were subjected to statistical analysis (SAS, 2009).

The effect of botanical extracts on seedling height and the number of leaves: Following the protocol of soil inoculation, 3 viable millet, sorghum and sesame seeds were separately planted in steam-sterilized potted (30-cm-diameter plastic pots) soil (2.5 kg of soil). The pots were completely randomized and replicated three times. After 24 hours, seeded pots were separately inoculated with 5 ml of PN, FA and NL crude extract, while sterile distilled water was used as the control (that is, negative control). NPK chemical fertilizer (15-15-15) was applied at a recommended dosage as positive control. Watering was done throughout the experiment based on the requirement. Data on seedling height and number of leaves were obtained after 4 weeks and subjected to statistical analysis (SAS, 2009).

Results and Discussion

Both blotter technique and soil inoculation methods significantly ($p\leq0.05$) enhanced seed germination, though, with a comparative variation. The soil gave better germination than the blotter technique (Hassan et al., 2012). Apart from the

fact that germination bioassays are excellent tools (Hoagland and Williams, 2003), it also provides the basis for comparison (Haq et al., 2014) which enables to determine the best botanicals in this study.

Apart from millet, PN and NL significantly ($p \leq 0.05$) demonstrated the phyto-fertilizer effect on sorghum. Similarly, FA enhanced germination of millet and sorghum seeds more in comparison to sesame that experienced slight inhibition. Islam and Kato-Naguchi (2014) had a similar observation and reported that *Ocimum tenuiflorum* at a concentration of 30mg dry weight (equivalent extract mL^{-1}) relatively enhanced the germination of barnyard grass. PN demonstrated more pronounced positive effects on sorghum and sesame (Table 1). PN, NL and FA might have altered well-defined sequence of complex processes underlying a set of biochemical, physiological and morphological changes in millet and sesame (Bewley and Black, 1994; Bewley et al., 2013).

Table 1. The effect of extracts on crops (% seed germination, radicle and plumule lengths).

	Parameters	FA	PN	NL	CT
Millet	SG (%) - BT	99.5a (± 0.76)	66.8b (± 0.07)	66.5b (± 0.07)	33.4c (± 0.14)
	SG (%) - SI	99.9a (± 0.21)	99.9a (± 0.07)	99.9a (± 0.07)	33.2b (±0.14)
	RL (cm)	9.3a (± 0.35)	4.8b (± 0.07)	9.2a (± 1.98)	9.4a (±0.21)
	PL (cm)	4.1b (± 0.14)	2.6c (± 0.92)	5.5a (± 0.14)	6.4a (± 0.14)
Sorghum	SG (%) - BT	99.9a (± 0.14)	99.9a (± 0.21)	99.9a (± 0.14)	33.2b (± 0.14)
	SG (%) - SI	99.6a (± (0.57)	99.9a (±0.14)	99.7a (± 0.42)	99.5a (± 0.78)
	RL (cm)	9.7a (± 0.35)	8.9a (± 1.91)	5.8a (± 0.99)	9.3a (± 0.07)
	PL (cm)	5.9a (± 0.50)	4.9a (±0.99)	10.9a (± 3.25)	6.3a (± 0.07)
Sesame	SG (%) - BT	50.1c (± 0.07)	83.4b (± 0.14)	NG	100.0a (± 0.07)
	SG (%) - SI	99.5a (± 0.78)	99.4a (± 0.92)	NG	99.9a (±0.14)
	RL (cm)	4.6a (± 0.14)	4.5a (± 0.14)	NG	5.0a (± 0.64)
	PL (cm)	3.6a (± 0.14)	3.3a (± 0.35)	NG	3.8a (± 0.42)

Mean values of three replicates are shown. Mean values followed by different letters in the rows are significantly different ($\alpha = 0.05$). BT = Blotter technique, SI = Soil inoculation, PN = *Parquetina nigrescens*, FA = *Ficus asperifolia*, NL = *Newbouldia laevis*, CT = Control. NG = No germination/growth. SG = Seed germination, RL = Radicle length, PL = Plumule length.

The botanical extracts responded differently to seed germination (Chukwuka et al., 2014). Specifically, NL exhibited a laudable effect on sorghum and millet in comparison to sesame that was completely inhibited. Also, since sesame seeds treated with NL were unable to germinate, radicle and plumule lengths could not be determined. This suggests that NL may contain some phyto-toxic compounds

which might have interacted with millet and sesame seeds, thus resulting in inhibition of plumule length and seed germination (Table 1, Figure 1). Killani et al. (2011) documented a similar observation with rice husk extract on cowpea. They found out that rice husk extract at 2% concentration level completely inhibited germination of cowpea seeds. In addition, the inhibitory effects of *Picrorhiza kurroa*, *Asperagus racemosus*, *Ocimum sanctum*, *Valeriana wallichii* (Rawat et al., 2016) and *Ocimum basilicum* L. (Verma et al., 2012) on germination and seedling growth of seed crops are in line with NL on sesame seeds. The effect of NA on sesame germination is detrimental, which suggests allelopathy. Thus, it could be deduced that NA exhibited an allelopathic effect on sesame. Therefore, this suggests that NA possesses allelochemicals that might have affected the enzyme responsible for plant hormone synthesis, inhibition of nutrient and ion absorption with a direct effect on plasma membrane permeability (Fujii et al., 1991). The practical implication of NL on sesame (if used by farmers) will lead to seed/plant mortality.

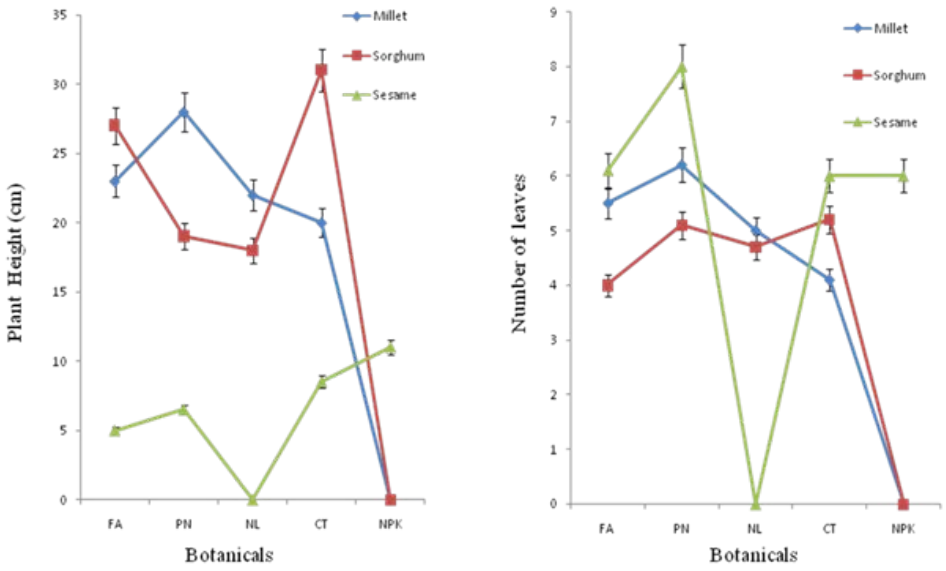

Figure 1. The effect of botanicals on the plant height and the number of leaves of millet, sorghum and sesame seedlings. PN = *Parquetina nigrescens*, FA = *Ficus asperifolia*, NL = *Newbouldia laevis*, CT = Control. SG = Seed germination, RL = Radicle length, PL = Plumule length.

The botanicals exhibited variation on the radicle length of the tested crops. FA was more effective on the crop radicle length in comparison to PN and NL. The effect of NL was outstanding on plumule length of sorghum while other botanicals showed no effect (Table 1). The report of Hassan et al. (2012) has stated that botanical extracts have the potentials to inhibit the emergence of sorghum radicle than plumule. The basis for comparison between this work and that of Hassan et al. (2012) can be linked to the choice of botanicals. Obviously, different botanicals possess different phyto-chemicals (Ntonifor, 2011) and have the potentials to react differently to seed germination and seedling growth.

Apart from the effect of PN on plumule length of millet that was inconceivable, other observable parameters were satisfactory. The PN extract had a stimulatory effect on early growth of millet and sesame (Tadele, 2014). High germination capacity and early growth of millet and sesame indicated that PN extract exerted a positive action on the germination of seedling growth (Tadele, 2014). PN also relatively enhanced the seedling height and the number of leaves (Akanmu et al., 2013) of the tested crops. This is in comparison to FA and NL with respect to seedling height (Figure 1). This suggests the possibility of using PN as phyto-fertilizer to enhance seed germination and seedling growth of millet and sesame. In comparison to NPK chemical fertilizer used as the control, the effects of botanicals on seedlings were comparatively different from that of NPK chemical fertilizer. Apart from NL on sesame, millet and sorghum seeds planted in botanically treated soil developed into seedlings while those planted in NPK treated soil were unable to develop into seedlings. This agreed with Carter (1967) who reported that chemical fertilizers including NPK can cause germination damage to certain crops if influenced by banding properties and other soil factors.

Conclusion

Apart from NL, PN and FA are potential phyto-ferilizers due to their effect on seed and seedlings of the underutilized crops. This study proposed that there may be phyto-toxic compounds in NL contributing to inhibition of sesame seed germination. The laboratory and greenhouse evaluation has helped to establish NL, PN and FA as potential phyto-fertilizers (though with variation) to millet, sorghum and sesame. Our future research is to investigate the importance of these results under field conditions and to determine the actual mechanisms involved in the differential effects of the botanical extracts on different species of underutilized food crops.

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BIOLOŠKO ISTRAŽIVANJE SA POTENCIJALNO KOMERCIJALNOM SVRHOM ZANEMARANIH BOTANIČKIH SUPSTANCI KAO FITOĐUBRIVA ZA NEISKORIŠĆENE PREHRAMBENE USEVE

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R e z i m e

Botaničke supstance su deo bioloških resursa za održiv rast prehrambenih useva, međutim, neke botaničke supstance su zanemarene. Ova studija istražuje moguće uticaje zanemarenih botaničkih supstanci na izabrane neiskorišćene prehrambene useve (proso, sirak i susam) u Nigeriji. Tehnika filter papira i metode inokulacije zemljišta korišćene su da se procene najbolje botaničke supstance koje se mogu koristiti kako bi poboljšali klijanje semena i rast rasada neiskorišćenih prehrambenih useva. Sa izuzetkom *Newbouldia laevis*, druge botaničke supstance poboljšale su klijanje semena svih istraživanih useva. *Ficus asperifolia* i *Parquetina nigrescens* značajno (p<0,05) su povećale dužinu radikule, dok je dužina plumule ostala nepromenjena. Posmatranje posebno pokazuje da je *Newbouldia laevis* potpuno inhibirala klijanje semena i rast rasada susama, dok je *Parquetina nigrescens* povećala visinu prosa i broj listova susama. Uopšteno govoreći, uticaj botaničkih supstanci na svaki usev bio je specifičan. S tim u vezi, ove zanemarene botaničke supstance imaju mogućnosti da poboljšaju i održe rasad neiskorišćenih prehrambenih useva u Nigeriji. Dalje istraživanje bilo bi sprovedeno kako bi se otkrile hemijske komponente kod testiranih botaničkih supstanci.

Ključne reči: đubrivo, rast, botanička supstanca, zanemareni i neiskorišćeni usevi, biljke.

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Zbornik
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Rezime
Rezime na srpskom jeziku (za radove napisane na engleskom jeziku) ili na engleskom jeziku (za radove napisane na srpskom jeziku) navodi se na kraju rada i treba da ima od 200 do 250 reči. Ispred osnovnog teksta rezimea, navodi se naslov rada, puno ime, srednje slovo i prezime svih autora i naziv i adresa ustanove autora. Naslov „Rezime“ piše se razmaknuto i centrirano. Nakon naslova sledi jedan razmak, a zatim tekst rezimea, uvučen jednim tabulatorom. Neposredno nakon teksta rezimea, navode se ključne reči, sa tačkom na kraju. E-mail adresa autora za kontakt navodi se ispod crte, pri dnu stranice.

Tabele
Tabele obeležene arapskim brojevima (1, 2, itd.) praćene naslovom treba da se nalaze na odgovarajućem mestu u tekstu, u fontu 9. Maksimalna širina tabela treba da bude 13 cm. One treba da budu jasne, što jednostavnije i pregledne. Treba izbegavati vertikalne crte, a broj kolona ograničiti tako da tabela ne bi bila preširoka. Takođe, treba izbegavati nepotrebnu upotrebu horizontalnih crta. Naslov tabele, poravnat po levoj i desnoj margini, sa tačkom na kraju, navodi se sa jednim razmakom iznad tabele. Ispod tabele treba dati detaljno objašnjenje skraćenica, simbola i znakova korišćenih u samoj tabeli. Svaka tabela mora biti pomenuta u tekstu.

Ilustracije
Svi grafikoni, dijagrami i fotografije treba da se nazovu „Slika“ (1, 2, itd.). Prilažu se na odgovarajućem mestu u tekstu. Grafikone i dijagrame treba uraditi fontom 9, u crno-beloj tehnici i sa maksimalnom širinom od 13 cm. Voditi računa da oni budu čitki i jasni i nakon redukcije veličine. Za svaki grafikon i dijagram treba obezbediti detaljnu legendu bez skraćenica. Fotografije moraju biti visokog kvaliteta da bi se tehnički mogle dobro reprodukovati. Prilažu se u „TIF“ ili „JPG“ formatu, u crno-beloj tehnici. Naslov ilustracije, poravnat po levoj i desnoj margini, sa tačkom na kraju, navodi se sa jednim razmakom ispod ilustracije. Svaka ilustracija mora biti pomenuta u tekstu.

Skraćenice i jedinice
U radu treba koristiti samo standardne skraćenice. Merne jedinice treba izražavati u internacionalnom sistemu jedinica (SI). Kod navođenja jedinica posle broja treba da stoji razmak (osim za % i °C). Skraćenice se mogu koristiti i za druge izraze pod uslovom da se ti izrazi navedu u punom obliku prilikom prvog pominjanja, sa skraćenim oblikom u zagradi. Vrednosti od 1 do 9 mogu se izražavati slovima, a ostali brojevi isključivo numerički.

Nomenklatura
Celokupna nomenklatura (hemijska i biohemijska, taksonomska, genetička itd.) mora biti usklađena sa međunarodnim kodeksima i komisijama, kao što su *International Union of Pure and Applied Chemistry, IUPAC-IUB Combined Commission on Biochemical Nomenclature, Enzyme Nomenclature, International Code of Botanical Nomenclature, International Code of Nomenclature of Bacteria* itd.

Formule

Sve formule i jednačine u radu moraju biti urađene pomoću programa „Word Equation“. Pri pisanju formula, radi preglednosti, ostaviti dovoljno praznog prostora oko same formule. Subskripti i superskripti treba da budu jasni. Prilikom pisanja jednačina treba dati smisao svih simbola odmah posle jednačine u kojoj se simbol prvi put koristi. Jednačine treba da budu numerisane arapskim brojevima, serijski u zagradama, na desnoj strani linije. Svaka jednačina mora biti pomenuta u tekstu kao Eq. (1), Eq. (2), itd.

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