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*Full Length Research Paper*

## **Structural and productive characteristics of Cowpea (*Vigna unguiculata*) under different water availabilities**

**Guimarães S. L.\*, Bonfim-Silva E. M., Moreira J. C. F., Silva S. L. S., Bosa C. K., Silva T. J. A. and Koetz M.**

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The cowpea has stood out on the national scene, opening up new perspectives and applications of a more suitable management to ensure quality product. Therefore, it is important to know the responsiveness to the levels of deficit and excess water in the soil. Thus, the aim of the present study was to evaluate the initial development of cowpea plants subjected to different soil water availabilities. The experiment was conducted in a greenhouse, in a completely randomized design with six water availabilities (40, 60, 80, 100, 120 and 140% of the maximum soil water holding capacity) with four replicates. Maintaining soil moisture was performed using gravimetric method. The results were submitted to analysis of variance and regression at 5% probability through Sisvar statistical program. The plant height, stem diameter, number of leaves, dry mass of shoots and roots were evaluated 35 days after implantation of the treatments. The water availabilities that provide better development and yield of cowpea BRS Nova Era stood in the range of 78.5 to 84.5% of the maximum soil water retention.

**Key words:** Brazilian Cerrado, *Vigna unguiculata*, water stress.

### **INTRODUCTION**

The cowpea (*Vigna unguiculata* (L.) Walp.) expands to the Cerrado region, in the North, Northeast and Midwest, where it is incorporated into production arrangements as second crop after soybean and rice and, in some places, as main crop (Freire, 2011). In the Cerrado region, especially when it is grown as second crop, the cowpea has a very competitive cost, a factor that has increased the interest of producers for the culture, as they may use the same practices of the previous crop. The expansion in the cowpea market opens up new perspectives and applications of a more suitable management to ensure quality product (Almeida et al., 2012).

According to Frota et al. (2008), the cowpea has short

cycle, low water requirement and ability to fix atmospheric N through N fixing bacteria. In this context, inoculation with effective rhizobia is a viable alternative to increase the productivity of the crop supplying the N needs by plants, lowering production costs and raising the income of the producer (Chagas et al., 2010). In general, for plant development, there is need for a balance between the water amount and the space occupied by the air in the soil. Legumes are usually sensitive to flooding and it may limit their growth because the nodes need O<sub>2</sub> to maintain the aerobic respiration necessary to supply the large amount of ATP, essential for nitrogenase activity (Thomas et al., 2005).

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In contrast, the soil water deficit can affect the root respiration and have adverse effects on plant development, with stomatal closure as the main response to stress, caused by the synthesis of abscisic acid, the consequent withering of leaves and the disability of the photosynthetic process affecting growth, subsequently (Sutcliffe, 1980). Yet, the plants submitted to the deficit have their stomatal conductance affected and this reduces the photosynthetic and growth rates, resulting in decreased production of shoots and roots (Santiago et al., 2001). Thus, the aim of the present study is to evaluate the development and yield of cowpea under different soil water availabilities.

## MATERIALS AND METHODS

### Location and experimental conditions

The experiment was conducted in a greenhouse at the Federal University of Mato Grosso, Rondonópolis Campus, located at latitude 16°27'44" S and longitude 54°34'48" W, 290 m. The soil was dystrophic Red Latosol, of medium texture, collected in the layer 0-0.20 m deep, in native Brazilian Cerrado area, and sifted through a 4 mm mesh sieve. Each plot consisted of pots of 8 dm<sup>3</sup> soil. The chemical and physical characterization of the soil was conducted in accordance with Embrapa (2017) and showed some characteristics. These characteristics include: pH (CaCl<sub>2</sub>): 4.1; P (mg dm<sup>-3</sup>) = 2.4; K (mg dm<sup>-3</sup>) = 28 mg dm<sup>-3</sup>; Ca (cmol<sub>c</sub> dm<sup>-3</sup>) = 0.3; Mg (cmol<sub>c</sub> dm<sup>-3</sup>) = 0.2; H (cmol<sub>c</sub> dm<sup>-3</sup>) = 4.2; Al (cmol<sub>c</sub> dm<sup>-3</sup>) = 1.1; SB (cmol<sub>c</sub> dm<sup>-3</sup>) = 0.6; CEC (cmol<sub>c</sub> dm<sup>-3</sup>) = 5.9; V (%) = 9.8; organic matter (g dm<sup>-3</sup>) = 22.7; sand (g kg<sup>-1</sup>) = 549; silt (g kg<sup>-1</sup>) = 84; clay (g kg<sup>-1</sup>) = 367.

The soil correction was performed by the base saturation method, up to the level of 60%, with addition of dolomitic limestone (PRNT = 80.3%). After the limestone incorporation, the soil samples were moistened to field capacity and incubated for 30 days. The experimental design was completely randomized, with five water availabilities (40, 60, 80, 100, 120 and 140% of the maximum soil water retention) and four replicates. The maximum soil water retention capacity in the pots was maintained by the gravimetric method, according to Bonfim-Silva et al., (2011). The basic fertilization with P and K was incorporated into the soil at planting. In accordance with the chemical analysis of the soil, 110 mg dm<sup>-3</sup> P<sub>2</sub>O<sub>5</sub> and 50 mg dm<sup>-3</sup> K<sub>2</sub>O were applied, using simple superphosphate and potassium chloride as sources, respectively.

The cowpea seeds (cultivar BRS Nova Era) were sown in pots and 5 days after the emergency, thinning was performed, leaving three plants per pot, which were grown for 35 days.

As a way of providing N to plants, 5 mL of the bacterial broth produced by multiplying the BR 3267 strain (*Bradyrhizobium japonicum*, recommended for inoculation in cowpea plants) was applied directly to the root zone of each plant. To obtain the bacterial broth, pure colonies of the rhizobia were multiplied for 24 h under constant agitation at 100 rpm in Dygs culture medium (Rodrigues et al., 1986). The water availability of all experimental plots was maintained at 80% for 15 days after germination, for plant establishment. After the application of the treatments and the stabilization of the respective availability to each treatment, two weighing of each experimental plot were performed daily to replace the water consumed by evapotranspiration, always in the early morning and late afternoon.

Thirty-five days after the implantation of treatments, it were performed the counting of leaves, the determination of height, stem diameter and the reading of chlorophyll content with chlorophyll meter. Later, cutting of shoots was performed close to the ground

and the roots were sieved and washed in a 2 mm mesh to prevent any loss. The shoots and roots were packed separately in paper bags and dried in forced air circulation oven at 65 ± 5°C until constant mass. Then, this material was weighed on a precision scale to determine the dry mass of shoots and roots.

### Statistical analysis

The results were submitted to analysis of variance and regression test at 5% probability, using the statistical program Sisvar (Ferreira, 2011). The variables dry mass of shoots and dry mass of roots were transformed by  $(X+1)^{0.5}$ .

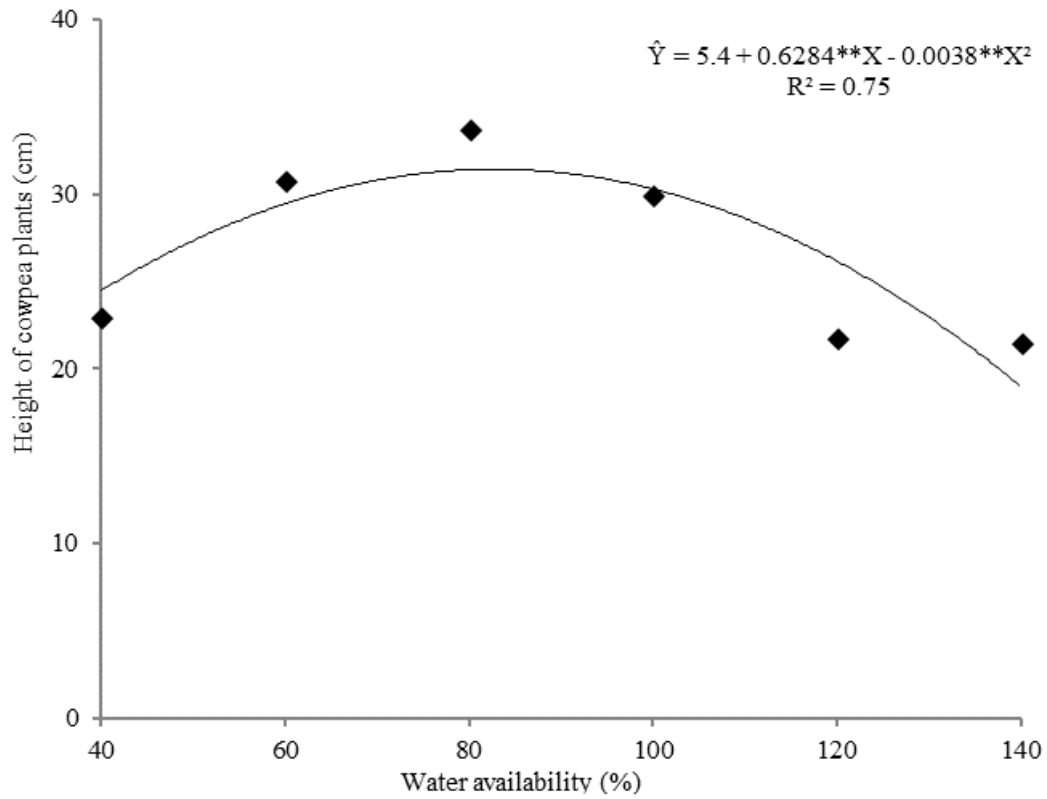
## RESULTS AND DISCUSSION

The water availabilities applied to the soil influenced all variables analyzed for cowpea. The growth of cowpea plants was directly affected by the soil water availabilities and the effects have been intensified in treatments where the plants were subjected to water stress, caused by both excess and deficit, a trend observed in all the variables analyzed. Plants reached the greatest height (29.08 cm) with 84.5% water availability, with subsequent decline. Water stress caused by the excess soil water, when compared to the maximum height obtained, it promoted 40% reduction in plant height; yet the deficit promoted a reduction of 22%, indicating the increased sensitivity of the culture in water excess conditions (Figure 1).

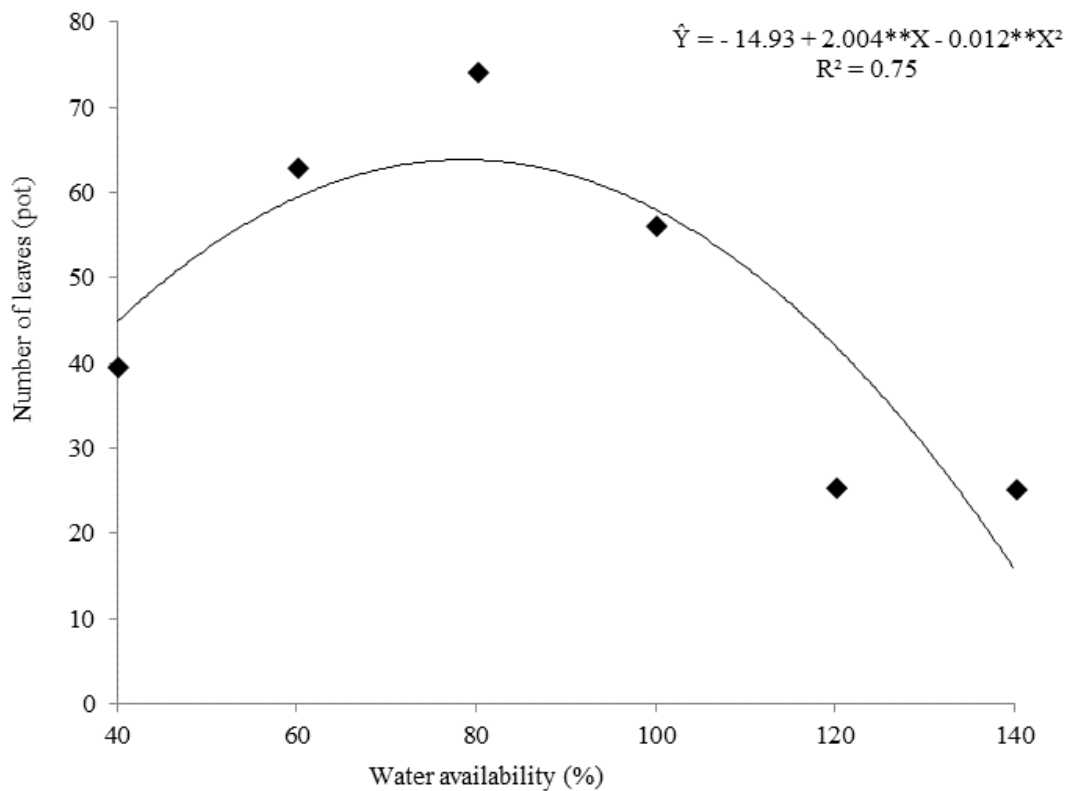
Marin and Santos (2008) evaluated the growth of pigeon pea (*Cajanus cajan* (L.) Mill sp.) under water deficit, found marked reduction in plant growth with increased water stress. Notwithstanding, the results found in the literature can be divergent, because in some cases there was a reduction plant height due to the fact that water stress reduces the turgor of the cells and consequently, their growth (Costa et al., 2008); just as there may be etiolation of plants when subjected to these conditions (Aguiar et al., 2008).

The production of leaves showed a quadratic adjustment to the levels of water availability, where the maximum production of leaves (69 leaves) was obtained in the water availability of 83.5%. The highest water deficit (40%) afforded a production of 46 leaves, representing a reduction of 28.1% in relation to the maximum production. The excess water in the treatment with 140% water availability provided the production of 30 leaves, corresponding to a 53.1% reduction compared to the greatest production (Figure 2). These results show the greater sensitivity of cowpea to excess water conditions.

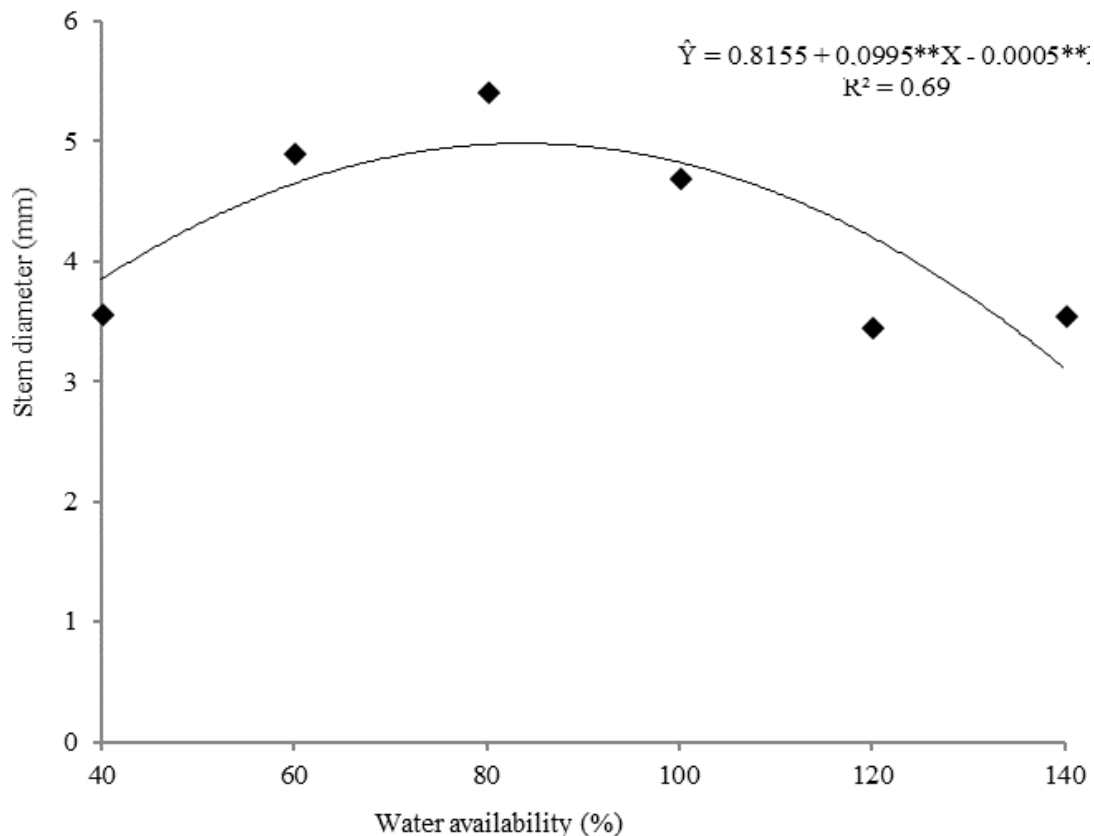
These results are related to the fact that the leaf growth depends mainly on the cell expansion because, once it is inhibited, there may be slowness of leaf expansion in the early development of the water deficit (Taiz and Zeiger, 2009). According to Larcher (2000), the reduction in the number of leaves of plants under water stress can be justified by increased endogenous production of hormones, such as abscisic acid and ethylene, since in



**Figure 1.** Height of cowpea plants, grown under different soil water availabilities.



**Figure 2.** Number of leaves of cowpea plants, grown under different soil water availabilities.



**Figure 3.** Stem diameter of cowpea plants, grown under different water availabilities.

the presence of these compounds there is greater senescence and leaf abscission.

Under water stress conditions, the plants have physiological responses such as liberation of abscisic acid (ABA), which is a plant hormone induced by water stress; on the plant, ABA induces the closure of the stomata, thus reducing the excess water loss by leaves and consequently the absorption of water from the roots (Shinohara et al., 2014). The lack of O<sub>2</sub> accelerates the production of ACC (1-aminocyclopropane-1-carboxylic acid), which is the precursor of ethylene. ACC is translocated from the root to the shoot and, in the presence of O<sub>2</sub>, it is converted into ethylene, which promotes shoot elongation, epinasty, leaf abscission, senescence and chlorophyll loss (TAIZ and Zeiger, 2009).

Nascimento et al. (2011), studying the development of cowpea under different levels of soil water availability, obtained linear results working with maximum water availability of 100%. Under these conditions, these authors observed reductions in the number of leaves, when comparing the control (100% water availability) with the levels of 80, 60 and 40%, of approximately 11, 23 and 35%; showing that the culture has increased tolerance to water deficit during the growing season, and hence

greater sensitivity at the beginning of the reproductive period.

The reduction in the development of plants under water deficit conditions is more emphasized in the development of leaves than in the stem expansion, this is the result of a plant defense mechanism to limit water losses by transpiration (Silva et al., 2011). The results of this study prove such statements, where the reduction in the leaf production (28.1%) remained higher than in the stem diameter (19.8%). The larger stem diameter was found with the water availability of 83.9%, resulting in 4.98 mm (Figure 3).

Evaluating the tolerance of bean genotypes to drought, Gonçalves et al., (2015) observed a 21.16% reduction in the dry mass of stem in relation to plants receiving adequate irrigation, the reductions were more pronounced for the fresh mass of stem, which was reduced by 48.65% in plants subjected to water deficit. Under flooded conditions, Fante et al. (2010) reported the formation of adventitious roots and stem hypertrophy in soybean plants. The lack of oxygen for prolonged periods leads many species to develop adventitious roots, aerenchyma and to the formation of cortical or peridermal cracks in the stems, resulting from hypertrophy (Batista et al., 2008). In flooded treatments, it was remarkable the emission of



Figure 4 (A and B). Cowpea plants subjected to treatment with 140% water availability.

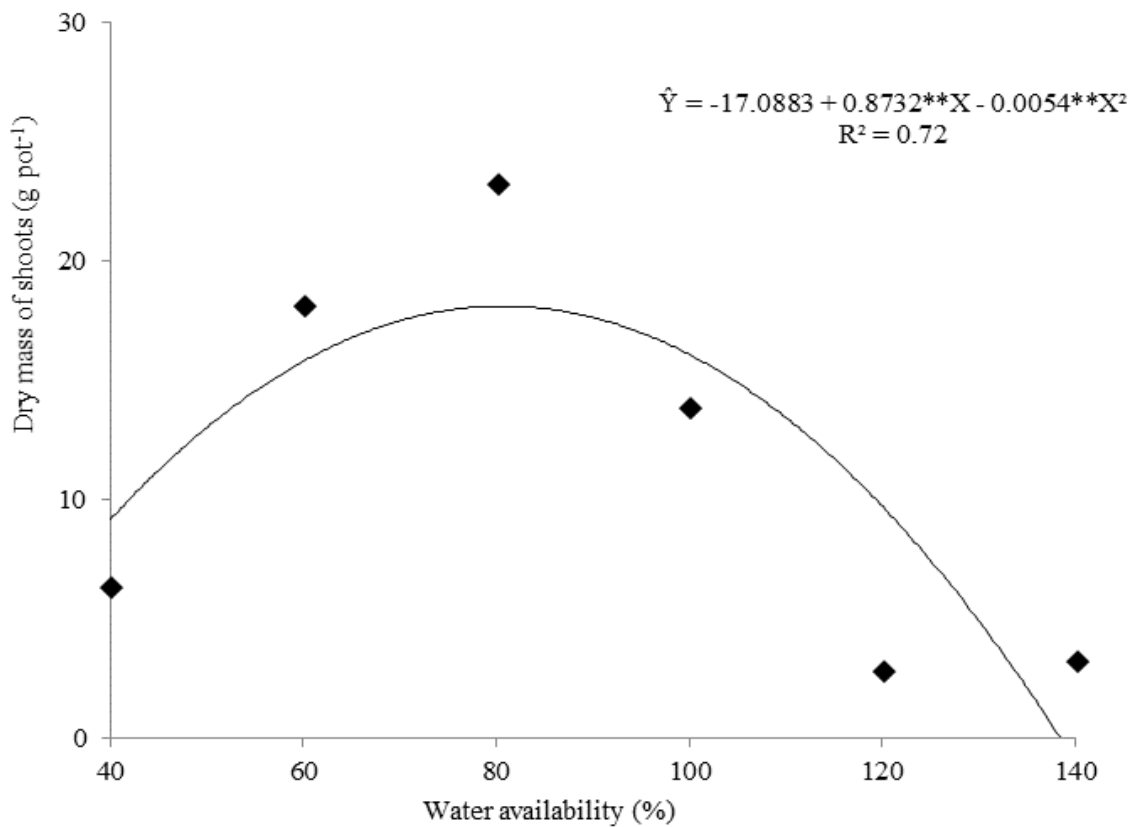
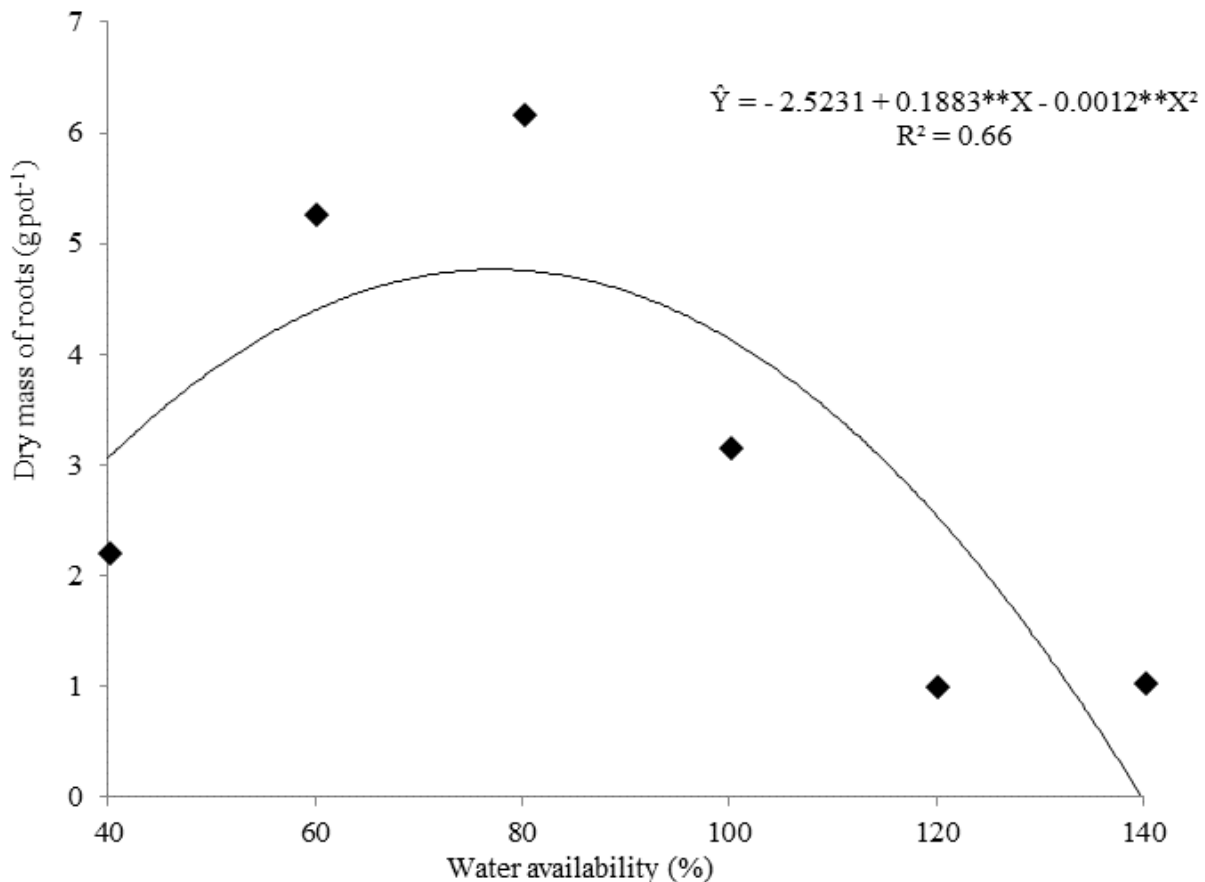


Figure 5. Dry mass of shoots of cowpea plants, BRS Nova Era, grown under different water availabilities.

roots in the soil surface and the crack in the stem, resulting from hypertrophy (Figure 4A and B).

The production of dry mass of shoots of the cowpea was influenced by the soil water availabilities, being adjusted to the quadratic regression model, where the

greatest production occurred with the soil to 80.6% of the maximum water holding capacity (Figure 5). The limitation in the growth of plants, imposed by the low availability of water, is mainly due to the reduction of carbon balance in the plant (Flexas and Medrano, 2002).



**Figure 6.** Dry mass of roots of cowpea plants, grown under different soil water availabilities.

In response to water stress, the plants close their stomata, in order to avoid excessive water loss through transpiration, but in parallel, there is a drastic reduction in CO<sub>2</sub> absorption, leading to a sharp decline in photosynthesis, which consequently interferes with the production of plant biomass (Alscher and Cumming, 1990). Decreases in the production of dry mass of shoots were observed by Freitas et al. (2014) and Mendes et al. (2007) in cowpea under water stress.

It is noteworthy that the development of cowpea plants is more affected by the excess soil water than by the deficit. Fante et al. (2010), evaluating 3 soybean cultivars (BRS 267, BRS 257 and BRS 213) under flooded conditions, observed average reduction of 55% in the production of dry mass of shoots. Under these conditions, respiration and plant metabolism are reduced because of oxygen deficiency in the soil, causing symptoms that initially appear as withering and leaf chlorosis, heading to stem hypertrophy, morpho-anatomical changes, decreased growth and root death (Kramer, 1983).

Evidencing the symptoms described, the dry mass of roots was influenced by the different water availabilities applied to the soil, reaching the maximum yield of 4.86 g pot<sup>-1</sup> with 78.5% water availability, nonetheless, it reached

the minimum point under a flooded condition (140% of the maximum water retention capacity), where there was practically no root growth (Figure 6). The water stress caused by water deficit in the treatment with 40% water availability was reduced by 36.6%, when compared to the maximum root production. Work done, especially under water stress conditions, demonstrate the sensitivity of legume species as beans (Custódio et al., 2009), peanuts (Duarte et al., 2013) and soybean (Firmano et al., 2009). The flooded condition of the soil (120 and 140% of the maximum retention capacity) provided significant reduction in the dry mass of roots. It is likely that after the application of treatments, the root system paralyzed its development, as reported by Palta et al. (2010), where root growth was paralyzed during the flooding, which resulted in a 67% reduction compared to the well-drained condition.

In the bean crop, the soil water availability is a major factor for plants to have a good development. Thus, knowledge of the ideal water availability for the development of plants is very important, since it is an important factor in decision-making regarding the management to be adopted, once, as seen in the results; the variation in the soil water availability directly affects

the vegetative development of cowpea.

## Conclusion

The water availabilities that provide better development and yield of cowpea, BRS Nova Era, stood in the range between 78.5-84.5% of the maximum soil water retention capacity for the development of cowpea. It was affected most by the water stress caused by excess water than by deficit conditions.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Constraints to private seed sector development in Ethiopia: A case study**

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The purpose of this assessment was to identify, analyse and document major policies, laws, regulations, administrative practices, governance and institutional setups that constrained private seed sector investment in Ethiopia and identify evidence based alternative solutions to improve the supply of certified seed to small holder farmers and livelihoods in the country. The study was conducted in 2017 in Oromia, Amhara, SNNPR and Tigray regions. Both primary and secondary data that comprise quantitative and qualitative data sets were used. Primary data collected using on 21 domestic and international private seed companies, 22 Key informant interviews, and 12 focus group discussions were conducted at various levels. The results of the analysis showed that there are problems connected with policy environment, institutional and administrative bottlenecks. Based on the result of the survey, 89.5 and 82.7% of the respondents replied there is inadequate marketing system and inefficient market access, respectively; 84.2% said availability of poor quality of early generation seed (EGS), and 68.4% limited availability of EGS; 71.4% respondents replied there is inadequate government support in terms of finance, and capacity development; 61.9% inadequate access to fertile land and suitable irrigable land. Hence, to improve the private seed sector investment in Ethiopia there is a need to develop a clear policy and directives across the seed value chain. In general, for the private sector to develop in the country there is a need to foster a stepwise reduction of government intervention in private seed production to ensure a level playing field between the public and private sector producers to attract more private companies to the seed sector and expand farmer choice.

**Key words:** Seed systems, seed policy, early generation seed (EGS) multiplication, seed production, public and private sectors.

## **INTRODUCTION**

Ethiopian agriculture contributes 36% to the GDP, 75% export and 80% employment (World Bank, 2017). Since the 1990s, the Ethiopian government has formulated and implemented the policy framework, known as agricultural development led industrialization (ADLI), with agriculture

as a primary stimulus to increase agricultural output, employment, and income of the people. According to ADLI, the agricultural sector should turn Ethiopia into an industrialized economy (MoFED 2013).

The target for improved seed supply is to meet 35

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million tons of certified seed that needs to increase from current production levels by about 18 million tons. Therefore, the primary deliverables to resolve seed system bottlenecks in growth and transformation plan (GTP-II) are to attract investment and develop a vibrant and competitive seed sector and strengthen regulatory capacity, structural and legal frameworks to meet international standards. Improved seed is one of the most important inputs for improving crop production and productivity. Its contribution is high when it is available in demanded quality and quantity at the right time and for the right price (Louwaars and De Boef, 2012).

Over 90% of the crops in developing countries are still planted with farmers' varieties and farm-saved seed (Almekinders et al., 1994; Almekinders and Louwaars, 1999; Maredia et al., 1999; World Bank, 1998). Farmers often cultivate several varieties of the same crop spreading across various plots. Furthermore, farmers may sub-optimally use improved varieties because they may not always use genetically pure seeds of improved varieties (Dawit and Zewdie, 2015). As a result, large international seed companies concentrate on those countries with large commercial seed sectors, often focusing on high-value crops grown by larger farmers in more favorable areas, that is, targeting those who are best able to pay for their seed.

In the past few years, several significant milestones have been achieved towards building a dynamic, efficient and well-regulated seed system in Ethiopia. A new seed sector development strategy has formulated by MoALR where systemic bottlenecks have been identified and key interventions have been formulated. Among these availability and quality of early generation seed (EGS) has been identified as one of the major constraints of the national seed sector (Abebe et al., 2016). The modest growth of the private sector in seed production is contributing towards the sustainability of the seed industry and in reducing the burden on the public. The development of new improved varieties and technologies by federal and regional research institutions continues to equip farmers with tools that improve productivity and livelihoods (Fikre, 2017).

In Ethiopia, the role of different actors in the seed sector has been clearly undefined. There was an attempt to develop a national seed industry policy for Ethiopia in the early 1990s. However, from the time the national seed industry policy was drafted in 1992, it is not known as to what extent the private sector companies are involved in seed production and marketing and how they are faring. Still, the public sector plays the greatest role in the system including regulating access to genetic resources, variety development, variety release and registration seed production, seed distribution and marketing. It is also responsible to monitor and regulate seed certification, variety protection and phytosanitary/seed quarantine control. The private sector's engagement in the seed sector is limited only to producing certified cleaning and delivering of Hybrid Maize seed to end

users. Some are also engaged in producing basic seeds for their seed multiplication. However, the modest growth of the private sector in seed production is contributing towards the sustainability of the seed industry and in reducing the burden of the public sector. Even though, there is a policy directive encouraging the participation of private sector actors, informal price controls are set by the Government in specific areas, like the production and marketing of hybrid maize. To this effect, there is therefore a need to conduct review that focuses on assessing the performance of the private sector and challenges facing in the national seed systems as this will help determine if the seed policy has achieved its intended purposes of facilitating different activities in the seed systems. Therefore, the objectives of this study was to identify, analyze and document major policies, laws, regulations, administrative practices, governance and institutional setups that constrained private seed sector investment in Ethiopia and identify evidence based alternatives solutions to improve the supply of certified seed to small holder farmers.

## MATERIALS AND METHODS

In line with the overarching objective of the study both primary and secondary data that comprise quantitative and qualitative data sets were used. A total of four methods were used such as document review, private seed sector survey, key informant interview, focus group discussion at federal, Oromia, Amhara, SNNPR and Tigray Regions. Data source targets were selected a broad range of stakeholders who were involved in the value chain of formal seed system. Hence, the outcome of this assessment would use for Government and its partners to improve the enabling environment to accelerate the development of the private seed sector in Ethiopian situations. The primary survey was conducted on 21 private seed companies (domestic and international) who are member of Ethiopian Seed Association located at Federal, Amhara, Oromia, SNNPR and Tigray Regions. Then, the data obtained through structured questionnaire were edited, coded and entered into SPSS (version 23) for analysis. Descriptive statistics such as percentage, frequency, mean and cross-tabulations were used to analyse the data.

A total of 22 Key Informant Interviews were conducted at various levels. The informants were purposively selected from the offices of Ministry of Agriculture and natural Resources (MoANR), Ethiopian Institute of Agricultural Research (EIAR), Ethiopian Seed Enterprise (ESE), Regional Public Seed Enterprises (RPSEs), Ethiopian Agricultural Investment Agency (EAIA), Ethiopian Agricultural Transformation Agency (ATA), ISSD Ethiopia, ICARDA, CIMMYT, FAO Ethiopia office, and public sectors in federal, Amhara, Oromia and SNNPR regional states. In addition, a total of twelve focus group discussions were done with 8-10 improved seed users' farmers for each session, and included their voice concerning problems and challenges they faced in the formal seed system at Amhara, Oromia and SNNPR.

## RESULTS

### Company profiles

In Ethiopia, there are more than 45 public and private

seed companies that are formally registered and producing different types of crops. Out of them 26 private and public seed companies are the members of ESA. Out of the total registered companies twenty one (80.8%) were identified for sampling. Out of these 33.3% of them were located in Amhara, 28.6% in Oromia, 14.3% each in Addis Ababa and SNNPR, while 9.5% of them were in Tigray regions. More than 75% of the companies are located in Amhara, Oromia, and SNNPR regions indicating these areas are suitable and potential for agricultural production. From the surveyed seed companies, some local private companies have modern pre- and post-harvest machineries, but some of them are unable to use them because of insufficiently available roads and electric power. The surveyed private seed companies used different machineries and facilities for running seed production, processing, storing and transporting purposes. Most of the respondent (76%) owned tractors to cultivate their land, 70% owned farm implements, 20% had combiners, 70% had threshers, 50% had cleaning machines, 45% had packaging facilities, 25% had labelling machines, 74% had storages to store their products, and 45% owned trucks to transport seed. It is well known fact that the seed industry requires huge capital investments, especially in infrastructure development and machinery purchase. Therefore, those who didn't have the required machineries and/or facilities would rent from other public/private service providers. Farm machineries for seed production and processing machines are equally expensive. Thus, to full fill those machineries and infrastructure the government has to consider tax free and set up credit mechanism for private seed companies. The total number of permanent and contract employees hired by the twenty one private seed companies were 4,116 and 2,163 per month respectively, and this would contribute for an employment opportunity in the local areas. This indicates that the growth of private sector in the country creates an opportunity to knowledge and technology transfer.

Some local private companies have modern pre- and post-harvest machineries, but they are unable to use them because of insufficiently available roads and electric power. The surveyed private seed companies used different machineries and facilities for running seed production, processing, storing and transporting purposes. Most of the respondent (76%) owned tractors to cultivate their land, 70% owned farm implements, 20% had combiners, 70% had threshers, 50% had cleaning machines, 45% had packaging facilities, 25% had labelling machines, 74% had storages to store their products, and 45% owned trucks to transport seed.

### **Policy environment in promoting private sector participation**

According to the data collected from the 21 private seed companies interviewed, 47.6% of the respondent replied

the investment policy was encouraging the private seed growers; whereas 52.4% of the respondents replied the investment policy was not encouraging the private seed growers. The main reasons according to respondents for discouraging private sector were lack of investment incentives (63.6%), no investment guarantees (36.4%), inadequate market access (72.70 %), and no tax exemption (18.2%).

In addition, 71.4% of the respondents replied the support of the government to the private sector is inadequate, 52.4% said inadequate access to public owned varieties of EGS, 57.1% believed there is a problem in policy implementation and enforcement at all level, 47.6% thought there is a challenge in varieties development and release system, 47.6% of the respondents replied that there is limitation in seed production and marketing, 33.3% of the respondents replied that there are inadequate access to germplasm and lack of seed import and export opportunities, 28.6% replied that there is seed regulation but it lacks proper implementation. Therefore, based on the information above, more than half of the respondents were challenged by inadequate support from the government side, followed by poor implementation and enforcement of the policy at all levels and inadequacy access to EGS. This is confirmed by key informants responding that in the country there is no clear policy direction on the role of private sector in seed industry development and investment.

In the study, it was also found that in collaboration with NARS some private seed companies have registered their own varieties. This was evident from the emerging seed companies that were relying on public developed varieties and those that have started their own variety development programs in collaboration with public research institutions. The notable constraints to this were i) the long bureaucratic process involved and the existing legal arrangement among private companies, MoANR and the public research institutions ii) the domestic private sector is under-capacity of the seed companies with regard to human, facility and capital resources. Furthermore, support in terms of variety purity maintenance should be offered to the private seed companies. For instance, in seed multiplication, private companies are accessing EGS from public research institutes that they give it to the seed out-growers for further seed multiplication. In addition, the findings also showed that none of the private companies received support in research and crop improvement and there is no clear guideline on how to establish private breeding institutions in the country.

### **Institutional and administrative bottlenecks that hinder the development of private sector**

The study revealed that there are institutional and administrative bottlenecks that hinder the participation of

private sector in the national seed system. These are inadequate supply of disease resistant varieties, lack of infrastructure and modern equipment, limited financial resources, inadequate implementation of policies starting from the grass root (*Wereda*) administrative up to the apex ministry level, and inadequate partnership and networking among stakeholders. Results showed that 57.1% respondents said that they have limited financial resources, 52.4% of each equally said there is lack of awareness of varieties, lack of modern equipment and poor road infrastructure facilities, 38.1% replied there is lack of disease resistance varieties, 38.1% of the respondent believed there is inadequate partnership and networking with stakeholders, 33.3% said insufficient support from *Wereda* level. Therefore, as per the collected data more than half of the respondents major institutional and administrative bottle necks are limited financial resources, lack of awareness of varieties, poor road infrastructures and lack of modern equipment, implying for a strong institutional and administrative support.

### **Constraints related to seed production and quality control**

Various constraints in the current seed system prevent private seed companies from increasing seed production and making it available to smallholders. The major constraints for the private sector are the fact that none of them do their own breeding institution. Therefore, they are relying on public institutions for EGS. The private seed companies obtain EGS from the research institutes and they produce certified seeds from it. Production of certified seeds is done in various ways; firstly at the company's own farm, secondly on private farms, and thirdly, on clustered farmers/farmer's associations fields. Based on the regulations, all seed fields must be inspected and certified before distribution. However, the current seed certification activity is weak both in coverage and quality of the operation. The number of the seed quality inspectors and their facilities do not match with the vast area of certified seed grown each year. Though, the capacity of inspectors is weak the regional inspectors are trying to monitor and supervise the seed production activities on both the company's own farms and on those of the contract farms.

Most private owned seed companies in the country lacks fertile and irrigate farm for their seed multiplication. The survey data showed that 29% of companies are multiplied seed on their own farm, 29% on out grower farms, and 43% grow both own and out grower farms. From this result one can understand that private seed companies had limited amount of land for their seed production. Based on the study, the seed companies are accessing early generation seed from different sources. 57.1% respondents replied they get from research

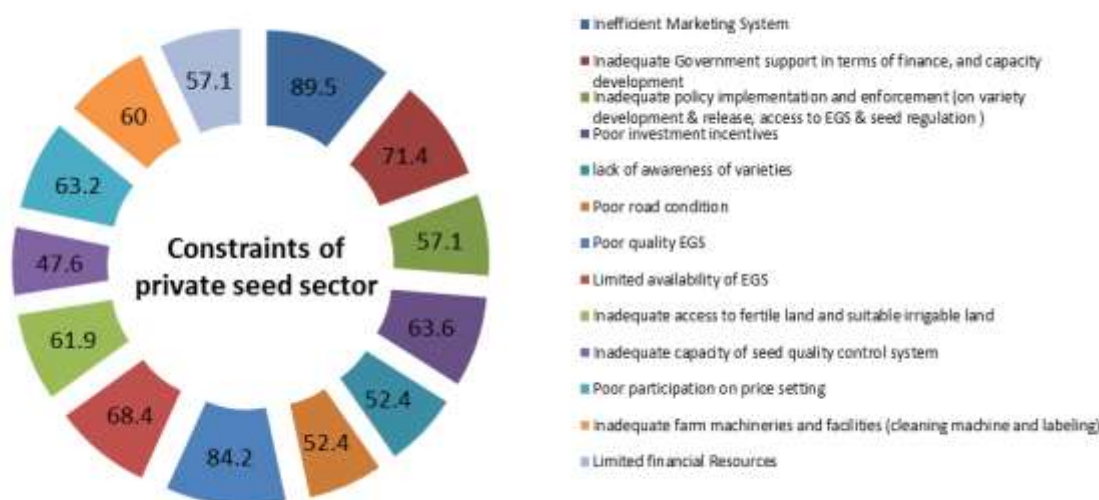
centres, 38.1% from public seed enterprises, 23.8% from BoANR, 14.3% from their mother company and 4.8% from Higher Learning Institutions (HLI). From the above data, one can understand that the public seed sectors are the major EGS suppliers in the country.

The identified private sector challenges connected to seed production and quality control systems are indicated as follows; 84.2% of the respondents replied that they are facing with problems of poor quality early generation seed, 68.4% with inadequate availability of EGS, 61.9% with lack of fertile and quality land with irrigation facilities, 61.1% of the respondent had limited skill on seed production, 50% of the respondent replied that they are challenged with quality assurance system on the process of seed production, 47.6% said they are limited on few crops and varieties, and 31.6% of the respondents replied they have scarcity of packaging facility during processing. From the above information collected one can understand that almost more than half of the companies are constrained with limited availability, choice and quality of EGS and fertile land with irrigation facilities for their certified seed production.

In this study, there were different challenges that the companies faced during seed quality control and certification. The major challenges of private sector in quality control and certification were inadequate capacity of seed quality laboratory (47.6%), inspectors did not inspect and report on time (33.3%), inaccessibility of farm to road (14.3%) and poor communication between seed companies and quality control offices (14.3%). During focus group discussion the farmers also complain that poor seed quality and sometimes low germination are the major problems faced after purchase of seeds.

### **Challenges related to seed marketing and distribution**

In Ethiopia, the international private seed companies have developed distribution network for the marketing of their products. Despite the generally very positive experience with the recent direct seed marketing pilots, the local seed enterprises relying on centralised public marketing system. Respondents were asked how the companies set their seed price, and replied that 47.6% set the price by assessing the market/ depend on the market condition, 38.1% depends on the production cost, 23.8% on public enterprise's prices and 19% based on the last year price. According to the information gathered, price determination is carried out in their own way differently across private sectors. However, the private seed companies who are setting their seed price on public seed enterprises are not considered on the price setting forum. Therefore, in the future considering the participation of private companies would have a positive impact on seed marketing. From the study report, it was revealed that the major challenges of seed marketing and



**Figure 1.** Priority constraints limiting the private seed sector performances.  
Source: Private Seed Sector Survey, 2017.

distribution were lack of an efficient marketing system (89.5%), lack of private participation in price setting (63.2%), absence of market competition (38.1%), absence of private agro dealers (28.6%), lack of price setting mechanism (42.9%), and limited resource capacity (47.6%).

### Priority constraints for the development of private seed sector

Based on the overall assessment ranges of constraints limiting the private seed sector performance in the country the major problems were identified and prioritized as indicated in Figure 1. Strength, Weakness, Opportunities and Threats of private seed sector are presented in Table 1.

### DISCUSSION

Ethiopia's seed sector is typical of the 'emergence stage' of seed sector development (Zewdie and Abebe, 2016). Seed production and supply is primary public sector, limited scale, with minimal competition from private sector producers. According to the Agricultural Transformation Agenda (ATA) annual report, the current average annual national seed supply of improved varieties for most food crops covers less than 10% of the total agricultural land area compared to 25% in many other African nations. Of the 10% of land, 87% is covered by seed that comes from public producers, 8% from multinational companies and 5% from local private companies. Supply of improved seed from public seed enterprises meets about 60% of government targets while private seed production accounts for less than 15% of supply, compared to 40%

in India and 20% in Tanzania (ATA annual report, 2015).

However, both the parastatal seed enterprises and local private sectors are not in a position to adequately meet country's seed demand. Therefore, multinational companies can play an increasingly important role in helping smallholder farmers acquire improved varieties of seed. The focus of multinationals should be on introducing new varieties to Ethiopia that do not directly compete with existing varieties. Seed sectors typically develop into four main optimally market archetypes, by crop (ATA, 2015). In this regard, countries such as Bangladesh, India and Turkey with more developed seed sectors adopt distinct roles for public and private producers by crop group.

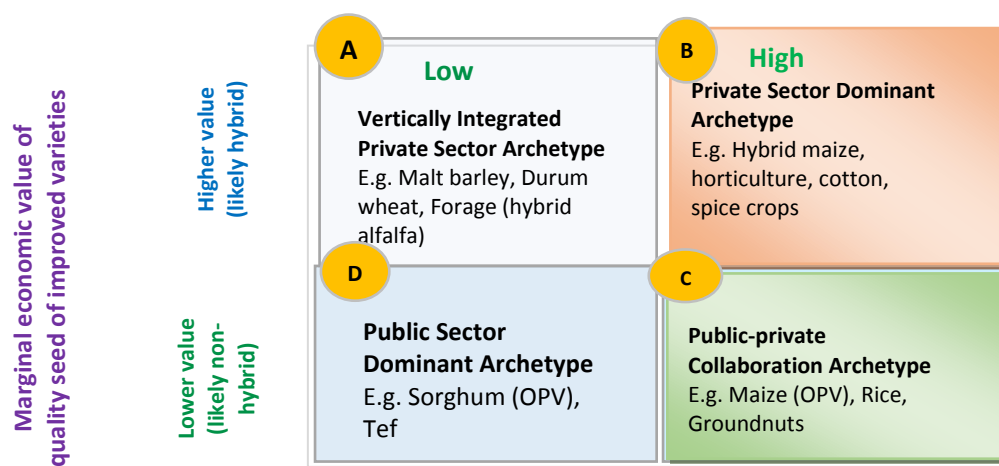
- (i) Vertically Integrated firms are suited to produce high value, low demand seeds
- (ii) Private Sector Dominant archetype is suited for high value, high demand seeds
- (iii) Public-private Collaboration is suited for low value, high demand crops
- (iv) Public Sector Dominant leads low value, low demand crops (Figure 2)

To improve the private seed sector investment in Ethiopia there is a need to develop a clear policy and directives on variety development and release. In many countries, public research takes the lead in areas such as pre-breeding, germplasm conservation, and crop and resource management. Experiences of other countries showed that they have simplified business registration, automatic and free variety registration.

During the study, there are practice constraints the government of Ethiopia should consider addressing to encourage greater private sector participation. The country's current variety release process has a number of

**Table 1.** SWOT analysis of private seed sector.

<b>Strengths</b>	<b>Opportunities</b>
(i) Agro climatic conditions:	(i) Seasonal coordination meetings on seed issues
(ii) Favourable agricultural policy and seed proclamation	(ii) Knowledge updating: Involvement of ESA for seed production
(iii) Organization running with profits	(iii) Opportunity to expand seed production in new potential areas:
(iv) Conducive environment for seed production/ multiplication	(iv) Strengthening the quality control lab and capacity building
(v) Better extension service	(v) Government support to provide lands by lease for seed production
(vi) Good coordination from seed certification agency and quality control officials:	(vi) Interstate outlets for direct seed marketing
(vii) Plant breeders rights proclamation	(vii) Expansion of irrigable land
<b>Weaknesses</b>	<b>Threats</b>
(i) Lack of government to encourage and organize seed production activity	(i) Disasters in drought and flood
(ii) Weak and long variety development and release process	(ii) Frequent changes in the policies
(iii) Lack of accurate planning	(iii) Threat of rejection due to isolation
(iv) Insufficient logistics and infrastructure (storage, transport, ware housing)	(iv) Threat of damage to seed due to improper storing
(v) Delay in packing of certified seed lots due to late arrival of results due to capacity problems	(v) Black marketing by suppliers during shortage of a popular variety
(vi) Poor marketing and distribution system	(vi) Limited supply of improved seed
(vii) Lack of loan from banks	
(viii) Lack of plant breeders rights implementation	

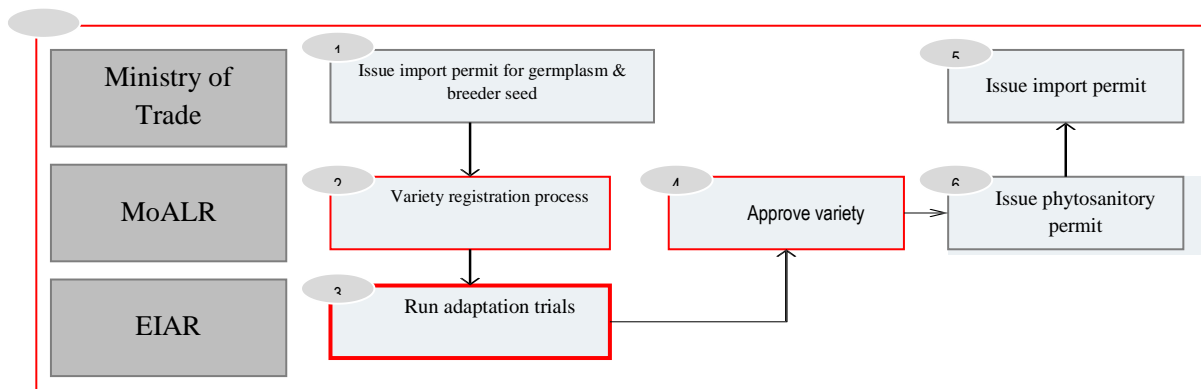


**Figure 2.** Demand for crops grown with quality seed of improved varieties. Source: ATA, 2017.

challenges, which discourage private sector companies from entering the country: firstly, the process by which a company applies for and manages the release of a new variety is logistically complex, requiring interaction with a number of different offices, in various geographic locations (Figure 3). This complex process is in contrast to India’s process which is centrally managed by the

Indian Council for Agricultural Research where companies simply have to interact with one office in one location to submit a variety for potential release. Therefore, the country should centralizing the variety release and other related administrative process (e.g., import permit application, phytosanitary permit application) into one nodal office; secondly, the pricing of





**Figure 3.** Variety release process challenges affecting the ability of the private sector  
Source: ATA, 2017.

variety testing is relatively high with a single test costing as much as \$10,000-15,000 USD, which is much higher with compared to a cost of \$200 USD in South Africa (ATA, 2015). This pricing gap should be improved and a clear and transparent pricing will be an important first step to reducing perceptions of bias; and finally, as the public sector is a key player in the seed industry, the objectivity of the variety release process and the variety release committee is in question. In the registration and adaptation trials may also be seed breeders, thus evaluating competitor product. Greater independence of the committee could reduce any perceived bias and needed streamlining the variety release process and increasing technical capacity throughout lower levels of government.

Plant breeders' rights, are important to private-sector involvement in breeding open-pollinating/self-pollinating crops (Manap et al., 2007; World Bank, 2006; Gerpacio, 2003; Loch and Boyce, 2003; Lyon and Afikorah-Danquah, 1998; Jaffee and Srivastava, 1994; Tripp, 1993; Pray and Ramaswami, 1991). Indeed, multinational companies commonly require that IPRs be in place as a precondition of their investment in research and development activities. Many authors recommend implementing plant breeders' rights consistent with UPOV 1978 or 1991 (e.g. World Bank, 1998; Louwaars, 2009). However, in Ethiopia even though the PBR law was recently approved by the parliament, it was not yet put for implementation. Therefore, weak guarantee of safeguards on protected varieties to international companies. This hinders participation of multinational seed companies to enter in the country. An efficient system that produce and distribute enough amount of EGS to public and private seed companies is also essential. Those seed companies wanting to commercialize varieties must be able to readily access sufficient quantities of source seed for growing on. Inadequate production of EGS by public institutions has been shown to be a constraint to diffusion of new varieties in sub-Saharan Africa (Tripp, 2000, 2006). Moreover, it is

essential to expand the private seed companies' access to EGS through issuing and enforcing an open and transparent application process with the clear goal of distributing EGS to all entities that meet a set of standards. Seed companies can improve the production of EGS in the case of limited public capacity.

Seed quality is a major determinant of seed acquisition behaviour (Zewdie and van Gastel, 2008), but is difficult for farmers to determine until the seed has been planted and is growing in their field (Minot, 2008). There are strong arguments for a continuing role for governments in setting and monitoring seed quality standards (Lyon and Afikorah-Danquah, 1998; World Bank, 1998; Kugbei et al., 2001; Minot, 2008), but implementation can increasingly be shared with seed producers themselves (Tripp and Louwaars, 1997). In Ethiopia, most of the private producers are complaining that the quality of EGS offered for sale is inferior in terms of physical and physiological quality. The internal quality control system for EGS production should be strengthened in human and physical capacity and premium prices paid for contract growers to encourage production of high quality seed.

Seed supply in Ethiopia is erratic for a number of reasons, with the key ones being major reliance on rain-fed agriculture, lack of cold storage for keeping carryover certified seed, and erratic supply of high quality foundation seed. Therefore, there is a need to develop support mechanism in provision of fertile and irrigated land to seed producer companies, build cold seed storages in different agro-ecologies to enable seed marketing and carryover certified stocks for sale in subsequent season that could lower production risks and the contractual agreement should be revisited and legally binding.

The government seed regulatory mechanism have different constraints like institutional and human resources capacity limitations, hence they are not able to respond on time to the request of private and public seed producers. Rigid application of seed regulations can

hamper the development of seed enterprises, as shown in Ethiopia (McGuire, 2005). Case studies on maize Brazil showed that certification of seed is valuable, ensuring that seed is of a specified variety and meets quality standards (Tripp, 2003), but excessively strict certification schemes can constrain development of the seed sector (Maredia et al., 1999; Kugbei and Zewdie Bishaw, 2002; Loch and Boyce, 2003; David, 2004; Minot, 2008; Smale et al., 2009). To improve the certification process allowing accreditation of private laboratories, private inspectors and university centres will have important role to lessen the burden on the public sector. The World Bank (1998) and Tripp (2006) also recommends voluntary seed certification based on government-set certification standards, with the public sector helping to develop the certification and seed testing capabilities of seed enterprises. The government should promote private sector through access to capital, credits and incentives, and support regional integration and harmonization to create larger markets. Support projects that directly work with private sector and strengthen their capacity. Governments may need to provide incentives to encourage development of seed marketing channels in less favourable areas (Tripp and Rohrbach, 2001). According to the study, availability of basic infrastructures in most of the local private sectors are lacking. This signals a direct need for sustained attention to critical infrastructure development and maintenance, and also an opportunity for public-private partnership in infrastructure investment.

The result of the study indicated that the emerging private sector companies in the country rarely have the experience or skills necessary to be able to ramp up and take over. These critical aspects could help to address: business planning, seed brand development, leadership and management skills, and technical and operational capacity etc. NARS breeders could work with emerging private seed companies to develop the most appropriate varieties for each agro-ecological need.

## Conclusions

It was found that it was actually difficult for a private company to access germplasm, the seed varieties and EGS from public institutions. There is a need for policy support and legal arrangement and direction that all the private seed companies have access to public bred varieties. This will make the seed industry competitive where the private seed companies can be made to bid for the public bred varieties and pay royalties. In such a case the public institutions involved in variety development become self-sustaining. In the other way private companies can also develop own competitive varieties.

The analysis of the seed system reveals that institutions do not govern the seed market in an optimal manner. To incentivize domestic as well as foreign

investments, well-designed and stepwise market liberalization is needed. Other countries experiences (India, Vietnam, Bangladesh, Kenya, etc.) also showed that seed market liberalization has enhanced supply and improved import export of seed. The direct seed marketing pilots in Ethiopia shows that the government has recognized the need for change and may slowly deregulate the market.

The policy should provide strategies to encourage the growth of the private seed sector in line with Government's overall strategy to encourage the private sector to assume command of the commercial components of the seed industry. The key linkage areas of the private seed sector with the public seed sector should be in the areas of the whole seed value chain (from germplasm access to marketing) adequate representation of private companies in relevant seed sector. Here, the ESA can play an important role in catalysing the linkages between all actors.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Introduction of the Sub1 gene into the Russian rice varieties using the polymerase chain reaction (PCR) methods**

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**Selection done using molecular markers has spread to all types of agricultural plants and animals. Marker assisted selection (MAS) is safe for ecology and food products consumed. The purpose of the study is to develop Sub1A gene (which determines tolerance to prolonged flooding) in the Russian rice varieties using DNA-markers and Polymerase chain reaction (PCR) analysis. A large volume of pre-breeding rice material (F2-F4, BC2F2) was created with tolerance genes for prolonged flooding, to control weed in rice agrophytocenosis. The forms with 120-130-day vegetation period were selected. An analysis of the co-inheritance of simple sequence repeat (SSR) markers Sub1Aq, Sub1C173, Sub1A203 with the Sub1 gene was carried out. It revealed the codominance of the selected microsatellites. They can be used to identify and visualize the allelic state of a given target gene in the breeding material. The recurrent parental rice forms, donors of the gene Sub 1 and the hybrids of F2 and BC2F1 population were tested on tolerance to prolonged flooding. The gene is effective in ensuring resistance to this stress factor within 2 to 3 weeks and it can be recommended for the breeding programs to develop modern genetic resources of rice tolerant to prolonged and deep flooding for weed control on rice fields.**

**Key words:** Polymerase chain reaction (PCR), rice, Sub genes, tolerance to flooding, weeds.

## **INTRODUCTION**

According to the occupied areas and a share in the gross volume of the yields rice is the third largest important food crop in the world. More than 3 billion people eat it and it provides 30% of food calories (FAOSTAT, 2016). In the Krasnodar territory, more than 80% of Russian rice

is produced on an area of 123-131 thousand hectares; in the Rostov region it occupies 8% of 14 to 15 thousand hectares. The main stress factors limiting the yield of this crop in the rice-growing regions are diseases, pests and weeds.

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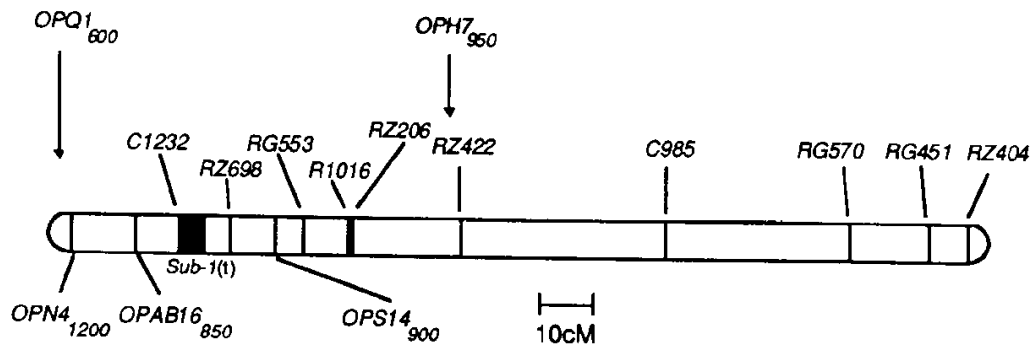


Figure 1. Localization of the Sub1 gene in the 9th chromosome.

Some studies show that weed invasion is a problem for the agricultural ecosystems, affecting production. It causes water stress, affects light and nutrients. Water stress is a problem for production as well as light. Many studies recently show that weed is to be managed with feed management. They explain fertilizer and seeds management. Seeding is very important for high yields and feed efficiency. Recent studies show that very high seeding are important to yield (Sevik and Cetin, 2015; Cetin et al., 2018; Tiwari et al., 2014; Torres et al., 2010; Goncharova et al., 2018; Kostylev et al., 2017).

Weeds compete for elements of nutrition, light and other factors with rice resulting in 20-50% of rice productivity shortage. Chemical protection of plants in some cases is neither efficient nor profitable (Kostylev et al., 2015).

Therefore, to eliminate weeds on rice fields, comprehensive solutions are extremely needed that could significantly reduce the use of herbicides, improve the quality of products and the profitability of the rice industry. In rice-growing, the production of sprouts from under the water layer, at which the malicious rice weeds (the *Echinochloa* species) die is considered to be the most environmentally friendly technology. However, its use causes a number of problems, primarily related to the high mortality of rice seedlings due to the lack of oxygen caused by the increase of the water level for weed control.

Therefore, an effective way to protect rice without herbicides is to grow varieties that are resistant to anaerobic germination and prolonged flooding (Dubina et al., 2017a).

The rice tolerance to flooding is controlled by the Sub1 (Submergence) locus, which was discovered by Xu and Mackill (1996) and Xu et al. (2006). It regulates the reaction to ethylene and gibberellin, which results in a restriction of the consumption of carbohydrates and the calmness of sprouts under water; it also promotes tolerance to prolonged and deep immersion. Expression of this gene increases with an increased concentration of ethylene (in flooding), which results in a decrease of gibberellin induced for plant growth.

Xu and Mackill (1996), using the population obtained from the cross-over of the rice-resistant rice line IR40931-26 (subspecies *indica*) and the susceptible line P154385 (*japonica* subspecies) in 1996, mapped the main QTL on the 9th chromosome near its centromere between the RLFP markers C1232 and RZ698 and gave them the designation Sub1 (Figure 1).

For the genetic mapping Xu et al. (2006) mapped this locus on the 0.06 morgand interval using a population of 4022 plants derived from the hybridization of the resistant variety FR13A (IR40931-26) (subspecies *indica*) and the unstable variety M.202 (subspecies *japonica*).

The Sub1 region is bounded by the CR25K and SSR1A markers and covers more than 182,000 base pairs. This interval codes three ethylene response factors, labeled as Sub1A, Sub1B and Sub1C, but only the Sub1A gene enhances plant tolerance to flooding (Mackill et al., 2012).

The International Rice Institute (IRRI, Philippines) worked on identifying the varieties tolerant to prolonged flooding for several decades (Hille, 1982). The Philippine colleagues have recently combined different resistance genes for prolonged flooding, including Sub1. On the genetic basis of the short rice variety 'Swarna', the first "super variety" 'Swarna-Sub1' with the Sub1 locus was developed. Based on the Sub1 sequent, there have been developed some specific molecular markers that greatly facilitate the marker assisted backcrossing system (MABC), which has been successfully introduced into the breeding process for the development and cultivation of "super-varieties" in Asia and Africa (Mackill et al., 1993; Septiningsih et al., 2009).

Due to MABC, the high productivity, as well as the consuming and nutritional quality of these "super-varieties" was maintained, which stimulates their demand among producers (Septiningsih et al., 2009). By 2011 eight such varieties had already been developed.

The purpose of the study is to develop rice breeding forms with the gene Sub1A, which determines tolerance to prolonged flooding, on the genetic basis of Russian varieties using DNA-markers and PCR analysis.

The development of rice varieties tolerant to this abiotic

factor of the environment is of great necessity today. Their cultivation will significantly reduce the costs of rice producers, as it will allow reducing the use of pesticides, avoiding ecosystem pollution as well as increasing the ecological status of rice growing in the Russian Federation.

## MATERIALS AND METHODS

The work was carried out in the laboratory of biotechnology and molecular biology of the FSBSI 'All-Russia Research Institute of Rice' and in the laboratory of rice breeding and seed-growing of the FSBSI 'Agricultural Research Center 'Donskoy''. Five varieties of foreign selection 'Khan Dan', 'BR-11', 'CR-1009', 'Inbara-3', 'TDK-1' (Vietnam) of the subspecies of *indica* with a vegetation period of 156-160 days have become the donors for the introduction of the Sub1A gene into Russian rice geneplasm (Azarin et al., 2016). In the south of Russia, it is preferable to cultivate varieties that mature not more than 125 days. As maternal forms for hybridization, the followings were selected: an early-ripening variety 'Novator' with a vegetation period of 105 to 110 days (developed by the All-Russian Research Institute of Rice); large-kernelled early ripening lines 'VNIIR-9678', 'KP-25-14', and rice lines 'PK-171 (*Pi-ta*)', 'KP-62 (*Pi-2*)' with blast resistance genes (developed by the All-Russian Research Institute of Rice), which allow obtaining rice geneplasm with complex resistance to biotic and abiotic factors of the environment. Hybridization of plants was carried out by pneumo-emasculation. Panicles were pollinated on the day of emasculation by the TVELL method (Los, 1987). For molecular genetic studies, a vegetative DNA was isolated from green leaves of the analyzed rice samples using the Murray CTAB method (Murray et al., 1980). Polymerase chain reaction (PCR) was carried out according to a standard procedure with 40-50 ng DNA, in a final volume of 25  $\mu$ l in the DNA-amplifier 'Tercik' (Kozhukhova, 1998). For establishing of the PCR, optimal conditions were selected and a protocol was drawn up where the output of the amplified product along with the minimum number of synthesized nonspecific DNA fragments was really high, that is initial denaturation is 5 min at 94°C (1 cycle). As for the next 35 cycles, denaturation step is 35 s at 94°C; primers annealing is 45 s at 60°C; synthesis is 30 s at 72°C. The synthesis lasts for 5 min at 72°C per 1 cycle. To identify the Sub1A gene, 76 flanking microsatellite SSR markers taken from the literature sources were used (Bailey-Serres et al., 2010; Xu et al., 2006; Yu et al., 2002; McCouch et al., 2001). Their sequence is presented in the NCBI genetic resources database ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)). Separation of amplification products with microsatellite markers was performed by electrophoresis in an 8% polyacrylamide gel. The electric field strength at electrophoresis was 3.9 to 4.5 V/cm. After electrophoresis, the gel was stained in a solution of ethidium bromide and photographed under ultraviolet light. To determine the length of the amplified fragments, a molecular weight marker was used (100 bp + 1.5 Kb + 3 Kb (SibEnZim) (0.05 g/L)) (Dubina et al., 2017b). Testing of recurrent and recipient forms of rice, hybrids, as well as weed-field forms of *Echinochloa* was carried out in the concrete tanks (1.0 mx 2.5 m). At the vegetative stage of rice plants, 5-6 leaves were deeply buried (50 cm of a water layer). After 14 days of flooding, the water was slowly pumped out. On the third day after the removal of water from the reservoir, the survived rice plants and weed species were counted.

## RESULTS AND DISCUSSION

The need to create genetic sources of agricultural plants,

offered for cultivation by environmental technologies, is constantly increasing. The Sub1A gene enhances rice tolerance to prolonged flooding, which can be used as an environmentally friendly factor for controlling weeds on rice fields.

At the first stage of the work, we searched for the information and made a selection of molecular (microsatellite) markers in the database [www.ncbi.nih.gov](http://www.ncbi.nih.gov) to identify and control the allelic state of the Sub1A gene in Russian rice varieties and donor varieties by PCR. Among 76 SSR markers tested for the presence of the Sub1A gene in the genotype of rice donor and maternal forms, 3 of the 4 microsatellites (Sub1Aq, Sub1C173, Sub1A203) showed the greatest level of polymorphism. Figure 2 shows the results of their testing. Sub1B showed no difference with the recessive alleles.

The selected SSR markers can be used in breeding programs to create rice varieties tolerant to this stress for identifying and visualizing the allelic state of the Sub1 gene in the hybrid rice plants developed by PCR.

At the next stage, the donors of Sub1A genes were hybridized tolerant to prolonged flooding with the varieties developed by the All-Russian Research Institute of Rice, which have high grain qualitative characteristics. There was obtained F<sub>1</sub> generation characterized by a high degree of sterility (90-95%) and brown color of flowering scales during maturation that indicates significant genetic differences between the parental forms. In the second generation, a wide spectrum of splitting was observed for all combinations in vegetative period, plant height, length and shape of panicle, number of spikelets, and awnness.

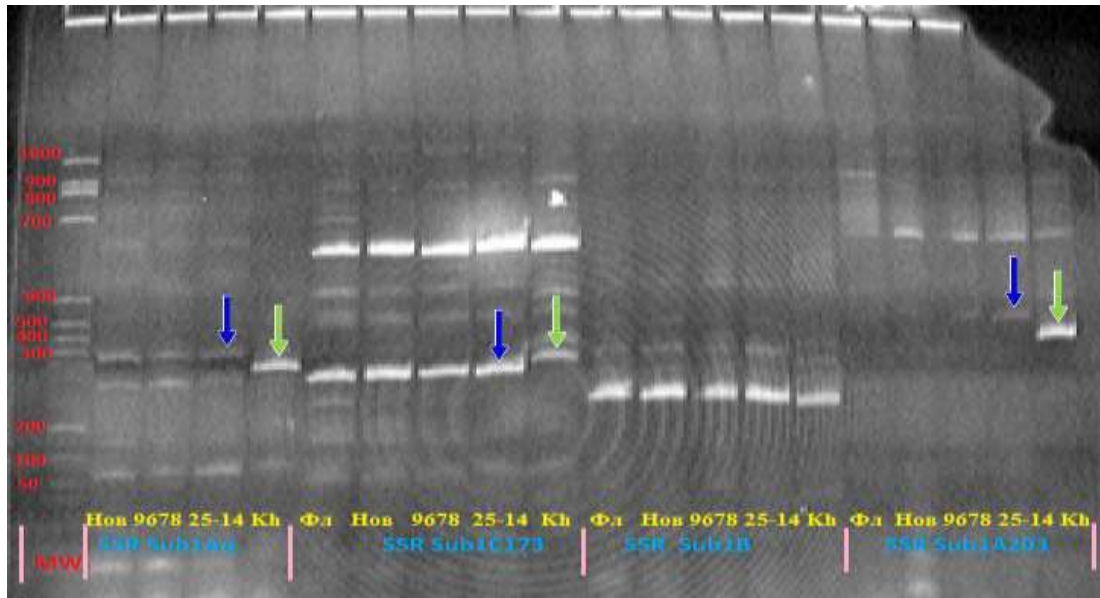
In the 'Novator  $\times$  Khan Dan' combination in F<sub>2</sub>, 184 plants were obtained, among which there were identified the samples combining the short height, resistance to shed its grain and fertility of the spikelets. Using DNA-marking, homozygous and heterozygous forms for the Sub1A gene were selected among these samples for further breeding (Figure 3).

Figure 3 shows that the hybrid plants N° 5 and 7 have a DNA profile and carry the allele of maternal form 'Novel'; the plant N° 11 has a specific allele of the Sub1A gene in the homozygous state.

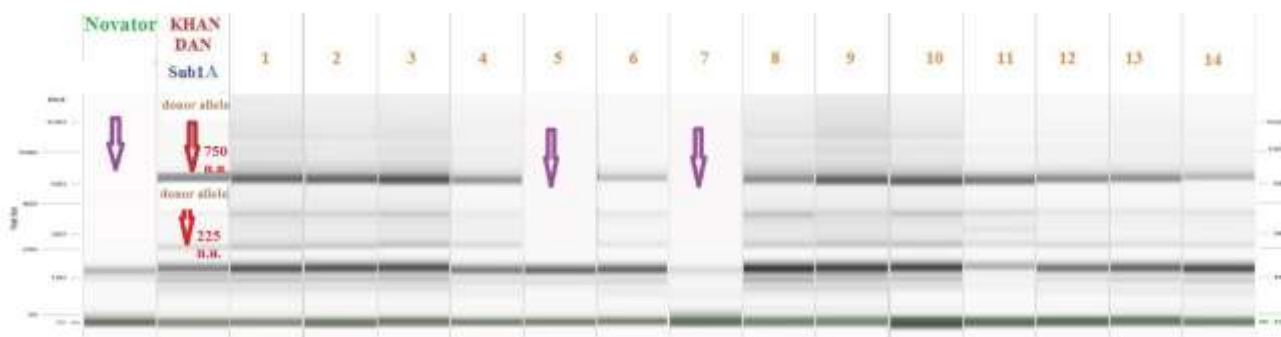
The molecular mass of the marker Sub1A203 is 203 bp. All other studied hybrid plants at this locus are heterozygotes. The plants that do not have a target gene according to the PCR analysis were rejected.

Based on the results obtained by the marker analysis of 184 plants, the following ratio was obtained: 39 plants carry a dominant allele in the homozygous state, 104 plants carry a dominant allele in the heterozygous, 41 plants carry homozygotes according to the recessive, which corresponds to a monogenic Mendelian split 1:2:1. This confirms the codominance of the SSR-marker Sub1A203, selected after approbation and its co-inheritance with the gene Sub1A.

Among the hybrids 'BR-11  $\times$  Novator' analyzed by the SSR-marker RM7481, Sub 1A gene (in the homo- and



**Figure 2.** Visualization of amplification of the products in SSR-loci *Sub1Aq*, *Sub1C173*, *Sub1B*, *Sub1A203*. MW is a molecular weight marker pBR322 / BsuR I (supplier is Helikon, Russia); Nov is an early ripening rice variety 'Novator' (maternal form); 9678 is a large grain rice line (maternal form); 25-14 is a large grain, early ripening rice line (maternal form); Kh is the variety 'Khan Dan', the donor of Sub1 gene; Sub1Aq, Sub1C173, Sub1B, Sub1A203 are the microsatellite markers for the Sub1 locus.



**Figure 3.** The results of PCR analysis on *Sub1A203* locus.

heterozygous state) was present in nine hybrids № 4, 7, 8, 9, 11, 12, 13, 18, 19 in a ratio of 9:11, although it should have been 15:5 with a monohybrid splitting.

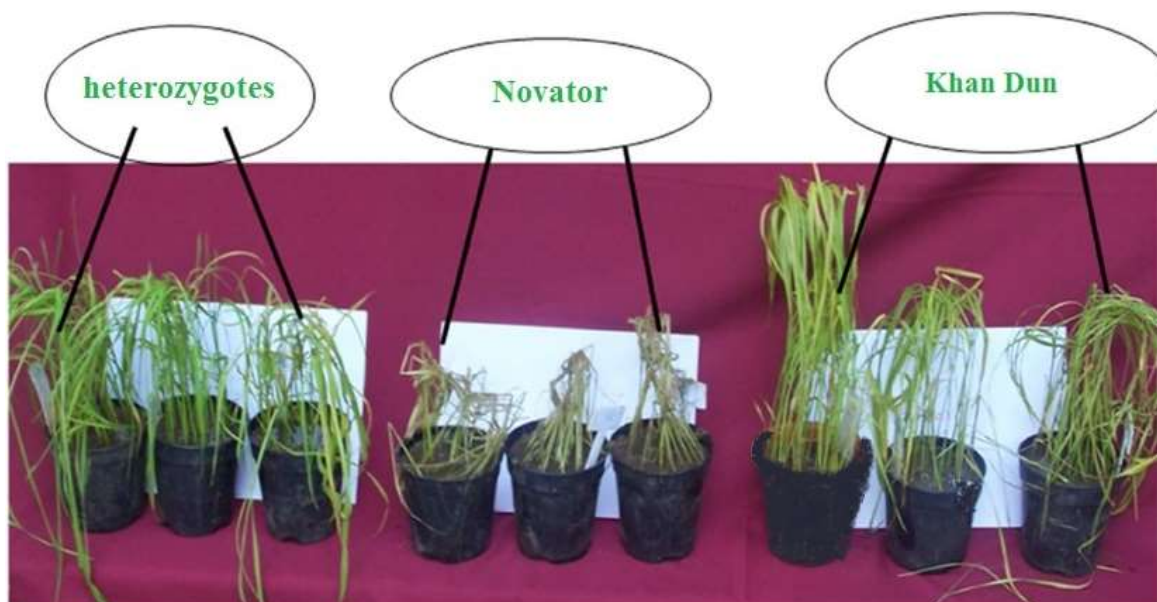
In a hybrid combination 'CR-1009×Novator', DNA analysis data showed splitting in  $F_2$  in a ratio of 18:2, that is, almost all selected plants had the Sub 1A gene. The hybrids 'Inbara-3×Innovator' and 'TDK-1×Novator' had a splitting of 14:6, or about 3:1, that is, closer to the Mendelian. Deviations in the splitting of two combinations can be explained by the effect of selection and the cohesion of genes.

Among 264 plants of five hybrid combinations, 198 plants were identified with the target gene. The selected samples were sown at the field belonging to the

Proletarskaya Experimental Station in the Rostov Region and on the lysimetric plots of the FSBSI 'ARRI of Rice' to evaluate economic characteristics.

The annual selection of basic plants from hybrid populations  $F_3$ - $F_5$  with a set of economically valuable traits was accompanied by a PCR analysis to identify the Sub1A gene in the laboratory of biotechnology and molecular biology of the FSBSI 'ARRI of Rice'. In 2017 more than 100  $F_5$  families were grown and propagated in each hybrid combination.

As the donor varieties have 156-day vegetation period in the conditions of the south of Russia, in  $F_1$  there were obtained hybrid plants with 140-day vegetation period, and in  $F_2$  there was a wide range of variability from early



**Figure 4.** The plants of rice parental and hybrid heterozygous forms  $F_1$  after testing for tolerance to prolonged flooding.

maturity ('Novator' type) to very late (at the end of September) flowering types. The forms with 120-130-day vegetation period have been selected for further work.

As earlier discussed, in the local rice cultivation area it is preferable to cultivate varieties with a vegetation period of no more than 125 days; therefore, work was done on the scheme of saturating crosses (backcrosses) in parallel with the earlier described strategy. The  $BC_1F_1$  and  $BC_2F_1$  generation has been obtained. This will allow creating rice lines with a set of economically valuable signs adapted to the growing conditions in southern Russia (with a vegetation period of not more than 125 days). According to the DNA analysis, heterozygous hybrid plants that will be involved in the further backcrossing were selected.

The next stage of this work was to test the donor variety 'Khan Dun' with the gene Sub1A, hybrids  $F_2$ ,  $BC_2F_1$ , as well as the Russian rice varieties and lines 'Novator', 'VNIIR 9678', 'KP-25-14' for tolerance to prolonged flooding. The test was carried out over the rice plants with 5 to 6 leaves in vegetation vessels in concrete tanks (lysimeters) under full flooding (50 cm water layer). Sowings were done by hand. The seeds of a hedgehog (chicken millet) *Echinochloa* species were also sown in the lysimeters. After 14 days the water was removed from the lysimeters, and on the third day after water discharge, continuation of the development or death of rice and weed plants were assessed (Figure 4).

From the experiment, the rice hybrids and the donor variety 'Khan Dun' with the Sub1A gene showed increased survival in the conditions of long-term and prolonged flooding in comparison with plants of Russian

varieties. The donor variety and hybrids restored their vital functions after flooding more quickly, producing more new leaves. The plants of the tested Russian rice varieties with 5-6 leaves were weak, depressed after reconstitution, and some of them died. The survived plants poorly formed the vegetative mass. The formed panicles were small in size (5 to 7 cm) and had 40% of sterile kernels. It was noted that on the experimental plots the weeds died under water.

Thus, a preliminary assessment of the obtained hybrids, Russian rice varieties and the donor of the Sub1 gene for tolerance to prolonged flooding has shown that this gene is effective for providing tolerance to this stress factor for 2 to 3 weeks and it should be recommended for breeding programs to develop modern genetic resources of rice, tolerant to prolonged and deep flooding, as a factor of weeds control.

## Conclusion

1. The information was searched and a selection of informative molecular (microsatellite) markers was made in the database [www.ncbi.nih.gov](http://www.ncbi.nih.gov) to identify the Sub1A gene by PCR in the obtained rice hybrids.
2. The testing of selected SSR-markers was done and their level of polymorphism in the contrasting rice samples was identified. Three SSRs with a high level of polymorphism on the basis of 'tolerance to prolonged flooding' were selected.
3. Hybridization of donor varieties of the Sub1A gene with Russian highly productive rice varieties and lines has



been done, that is early-ripening variety 'Novator', large-kernelled early-ripening lines 'VNIIR-9678', 'KP-25-14', as well as with the lines 'KP-171' and 'KP-62' with blast resistant genes *Pi-ta* and *Pi-2*, respectively. The generations F<sub>1</sub>-F<sub>4</sub> and BC<sub>2</sub>F<sub>1</sub> were obtained.

4. DNA-analysis of the obtained hybrids on the identification of the Sub1A gene was done. The plants having a target gene in the hetero- and homozygous state in the genotype were selected.

5. The co-inheritance of the Sub1 gene linked to the sign of 'tolerance to prolonged flooding' was studied based on PCR analysis and the codetermination of the selected SSR-markers was established.

6. The testing of the selection rice resources on prolonged flooding resistance during the vegetative experiment was done. The tolerant forms of rice were selected and approved for use in the breeding process to create new rice varieties with valuable agricultural characteristics.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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*Full Length Research Paper*

# The use of plant extracts in anthracnose control in species of *Heliconia* (*Heliconia psittacorum* cv. Golden Torch and *Heliconia rostrata*)

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The objective of the present study was to assess the anti-fungal activity of medicinal plant extracts on inhibition of *Colletotrichum gloesporioides* mycelial growth, sporulation and germination and on anthracnose control in *Heliconia psittacorum* cv. Golden Torch and *Heliconia rostrata*. Extracts of garlic, lemon grass, cinnamon, lemon balm, eucalyptus, ginger, mint, bitter melon and black pepper were used. The mycelial were assessed *in vitro* by measuring their growth in Petri dishes. Sporulation was assessed by counting spores in a Neubauer chamber. Germination was observed based on germination tube emission. In the *in vitro* tests, the inflorescences were treated with the plant extracts and inoculated with the pathogen 24 h later. The results showed that all the extracts presented anti-fungal activity, in greater or lesser intensity, compared to the control. The garlic extract resulted in the highest mycelial growth inhibition rate. Regarding sporulation, the bitter melon, ginger, mint, popcorn eucalyptus and bitter melon extracts were more efficacious, interfering in spore formation; while the ginger extract most reduced spore germination inhibiting germination tube emission. For the *in vivo* tests, it was observed that all the plant extracts tested were efficient in reducing lesion severity in the inflorescence, showing that the use of plant extracts may be a promising alternative for managing the diseases that affect helconia post-harvest.

**Key words:** Alternative control, fungi, *Colletotrichum gloesporioides*, tropical flowers.

## INTRODUCTION

Floriculture is one of the main segments of agrobusiness in Brazil, although the main consumer of flowers and ornamental plants produced in Brazil is the domestic Brazilian Market itself that accounts for more than 96% of the total (Sebrae, 2015). From 2014 to 2015, the volume

of Brazilian exports was only 21.9 thousand dollars according to the Ministry of Industrial Development and Foreign Trade, values much lower than those presented in 2004 and 2005, which were a total of 12.7 million dollars (Brasil, 2017).

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In 2015 the ornamental plant sector grossed more than R\$ 6 bilhões (Alencar and Galera, 2016), showing its size and importance in the Brazilian economy. In the same period, the planted area was approximately 15.000 hectares, due mainly to a recurrent increase in the area used for this activity in Brazil, as in 2012 and 2013 the area was estimated at about 11.800 and 14.000 ha, respectively (Neves and Pinto, 2015).

There are no recent studies in the literature on the productive chain in the state of Maranhão, Brazil, and the local production is still not very enterprising and focused essentially on supplying São Luís, the state capital. It concentrates on exploiting cut flowers and tropical foliage that include palm trees, bromelias, ferns, miniature roses and crotons (Sebrae, 2015).

The conditions of tropical ornamental plant cultivation, climate and planting density favor disease occurrence. Diseases reduce production and affect flower quality; becoming a bottleneck for quality tropical flower production, because they cause both direct damage, when they infect the inflorescences causing dark spots, and indirect damage, by reducing the photosynthesis area of the plant when they colonise the leaves (Sardinha et al., 2012).

Sardinha et al. (2012), surveyed disease occurrence in tropical flowers on São Luís Island and reported the presence of 16 disease causal agents, especially fungal and nematoid diseases.

Plant diseases are mostly controlled by agricultural chemicals, which results in high costs, environmental and toxicological risks and favors plant pathogen resistance to agricultural chemicals. Society's concern with dependence on toxic agricultural chemicals, that contaminate the environment, has led to the search for alternative control methods that are safe, viable and efficient in plant pathogen fungus control (Silva et al., 2010).

Control alternatives, including the use of biological and antagonistic plant extracts, have been studied, with significant advances for sustainable agriculture (Freitas, 2008).

Considering the potential of tropical flowers as a source of income for small and large producers and the presence of plant pathogens that can affect the yield of this activity, technologies need to be adopted that meet the requirements of these producers, especially in decreasing plant pathogens. Thus the objective of the present study was to assess the anti-fungal activity of crude plant extracts on the pathogen *Colletotrichum gloeosporioides* Penz., causal agent of anthracnose in *Heliconia psitacorum* cv. Golden Torche and *Heliconia rostrata*.

## MATERIALS AND METHODS

The experiments were carried out in the Plant Pathology Laboratory at the Agronomic Biotechnology Nucleus at the State University of

Maranhão (UEMA), MA, Brazil.

### Plant collection and plant pathogen isolation

Heliconia leaves with disease symptoms were collected during three technical visits to the Vassoural Agrucultural Pole, in the municipality of Paço do Lumiar, on São Luís Island, Maranhão, Brazil. The material collected was taken to the Plant Pathology Laboratory at UEMA for isolation and identification and the isolates were stored.

To isolate the plant pathogens, intermediary fragments of the lesions were cleaned with 70% alcohol, 0.5% sodium hypochlorite solution and washed twice in distilled, sterilised water. The fragments were then plated on Petri dishes containing potato dextrose agar culture medium (PDA) and kept at ambient temperature ( $25\pm 2^\circ\text{C}$ ) for fungal growth. The isolates were identified by observing the morphological aspects under a microscope and when necessary microcultures were used (Menezes and Assis, 2004). After identification, the isolates were placed in the "Prof. Gilson Soares da Silva" plant pathogenic fungus collection, with registration number MGSS114.

### Obtaining the aqueous extract

Extracts used from garlic (*Allium sativum* L.), black pepper (*Piper nigrum* L.) and ginger (*Zingiber officinale* Roscoe), were obtained from retail stores in São Luís; lemon grass (*Cymbopogon citratus* L.), cinnamon (*Cinnamomum zeylanicum* Blume.), lemon balm (*Lippia alba* L.) and mint (*Mentha piperita* L.), were purchased in public markets and eucalyptus (*Eucalyptus globulus* L.); while bitter melon (*Momordica charantia* L.), and neem (*Azadirachta indica* A. Juss.) were collected from the Paulo VI University campus at the State University of Maranhão (UEMA). The garlic and ginger extracts were prepared from bulbs and the other extracts were prepared from fresh leaves.

The proportion used was 200 g/L; the materials were first washed with distilled water and then ground in a blender for 3 min. The extract was filtered three times: using a plastic sieve, then through a glass funnel containing sterile gauze and lastly through nitrocellulose membrane with 0.22  $\mu\text{m}$  diameter pores, attached to a syringe. After filtering, the extracts were placed in a sterile dark recipients and kept refrigerated at  $4^\circ\text{C}$  until the tests.

### Plant extract assessment on *C. gloeosporioides* mycelial growth and sporulation

The plant extracts at 20% concentration were placed in PDA culture medium and poured into a previously autoclaved Petri dishes. Five mm diameter discs containing *C. gloeosporioides* structures were transferred to the centre of the Petri dishes and kept at ambient temperature ( $25\pm 2^\circ\text{C}$ ).

Mycelial growth was assessed by measuring the mycelial growth of the colony, establishing a mean of two measurements taken at two diametrically opposite points, until the control treatment had taken the whole of the Petri dish. The inhibition percentage of the mycelial growth (PIC) was determined from the results, according to Edgington et al. (1971):

$$\text{PIC} = (\text{Control Growth} - \text{Treatment Growth}) / \text{Control Growth} \times 100$$

Sporulation was assessed by evaluating the mycelial growth and 10 ml sterilised distilled water were added to each Petri dish. The colonies were scraped using a Drigalski handle to release the conidia, that were counted using a Neubauer chamber.

### Assessment of plant extracts on *C. gloeosporioides* conidia germination

To assess germination inhibition on *C. gloeosporioides* conidia provided by the plant extracts, a  $4 \times 10^5$  conidia.mL<sup>-1</sup> conidia suspension was prepared using a Neubauer chamber. Twenty microliters of the conidia suspension and 20 µL of each extract to be tested were placed on sterilised glass slides. The control consisted of the conidia suspension with no plant extracts. The slides were placed on Petri dishes containing two layers of moist filter paper and kept in BOD at  $25 \pm 1^\circ\text{C}$  for 9 h (Celoto et al., 2008). At the end of this period, a drop of lactophenol was added to interrupt spore germination. The germination percentage was determined by counting 100 spores under a microscope, separating the germinated from non-germinated spores. A spore is considered germinated when it presents a germination tube bigger or equal to its width.

The spore germination inhibition percentage (PIG) was obtained from the results by the following formula:

$$\text{PIG} = (\text{Control growth} - \text{No. of germinated spores the treatment}) / \text{Number of spores germinated in the control} \times 100$$

### *In vivo* assessment of plant extracts for anthracnose control in *H. psittacorum* cv Golden Torch and *H. rostrata*

After collection, the cut helconia were taken to the Plant Pathology Laboratory at the State University of Maranhão and selected with uniform colour and size and no mechanical injury. The flower stems were disinfected by washing with 10% (v/v) sodium hydrochlorite solution for 2 min, then washing in running water. After drying, the stems were submitted to the treatments by pulverisation with the plant extracts at 20% concentration and the addition of Tween 20 (0.02% v/v).

After applying the plant extracts, the plants were kept in a wet chamber for 24 h and then inoculated with *C. gloeosporioides* by placing a disc of pure fungus culture in PDA culture medium on the flower stem lesions and each stem was inoculated at three points. Assessment started three days after inoculation, when the severity was assessed by measuring the lesion size.

The experimental unit consisted of one flower or inflorescence. The assessments were carried out by measuring the mean lesion diameter and establishing the mean of two measurements taken in diametrically opposite positions.

### Statistical analysis

A completely randomised block experimental design was used with 11 treatments and five replications, except for the *C. gloeosporioides* conidia germination assessment, where four replications were used. The data obtained were submitted to analysis of variance and the means compared by the Scott-Knott test at the level of 5%.

## RESULTS AND DISCUSSION

### Effect of plant extracts on *C. gloeosporioides* mycelial growth and sporulation

The result of the analysis of variance indicated differences in the plant extract anti-fungal activity on *C. gloeosporioides* for both mycelial growth and sporulation (Table 1). At the end of the assessments it was observed that the plant extracts tested presented anti-fungal

activity, in greater or lesser intensity, compared to the control, based on the fungus colony diameter.

The garlic extract treatment presented the best result with 50.37% *C. gloeosporioides* mycelial growth inhibition (Table 1), corroborating Venturoso et al. (2011) who assessed the inhibitory effect of plant extracts on plant pathogens and observed that extracts of garlic, clove and cinnamon presented fungitoxic properties and inhibited mycelial growth of the plant pathogens tested (*Aspergillus* species, *Penicillium* species, *Cercospora kikuchii*, *Colletotrichum* species, *Fusarium solan*, *Phomopsis* species). Mycelial growth inhibition by natural antimicrobials is due to the presence of bioactive substances found in plants (Chiejina and Ukeh, 2012; Silva et al., 2014).

The potential fungitoxic effect of crude garlic extract on mycelial growth was also observed in fungi that cause anthracnose in strawberry plants (*Colletotrichum acutatum*) (Almeida et al., 2009) and on the causal agent of red rot in sisal (*Aspergillus niger*) (Souza and Soares, 2013). Nascimento et al. (2013) tested aqueous extracts and observed that bitter melon was efficacious in inhibiting mycelial growth of *Cercospora calendulae* at the (10000 mg L<sup>-1</sup>) concentration with 40% inhibition.

Although garlic extract resulted in less mycelial growth compared to the other extracts, when sporulation was observed, the extracts of bitter melon, ginger, mint, neem, eucalyptus, pepper and lemon balm were more efficacious in inhibiting *C. gloeosporioides* spore production (Table 1). However, bitter melon gave the biggest anti-sporulation effect that may have been due to production of bioactive substances such as momordicin, alkaloid, flavonoid, saponin, glycoside, phenol constituents, phenylalanine, arginine, lignan-calceolarioside, and triterpene-momordicine alkaloid zeatin (Martins-Ramos et al., 2010).

When spore formation is more inhibited, the product is more efficient. This is because the spores produce propagules that disseminate and infect the plant.

Several studies have indicated the use of plant extracts for fungal disease control. Simon et al. (2016) studied medicinal plant extracts to control *Diplocarpon rosae* and observed anti-sporulation effect using crude *Equisetum arvense* L. aqueous extract (EBA) and a commercial product based on fermented plant extracts, and further observed protein synthesis in the *D. rosae* mycelium in the treatment with EBAs of *R. officinalis*, *E. arvense*, and *Moringa oleifera*, the commercial plant oil-based product and a citrus matter-based product, respectively. Ferreira et al. (2014) observed an inhibitory of neem seed extract until the third day that reduced sporulation in *C. gloeosporioides* collected from papaya fruits.

### Plant extract effect on *C. gloeosporioides* conidia germination

All the plant extracts tested in the germination experiment

**Table 1.** Effect of different plant extracts on mycelial growth and sporulation of the fungus *Colletotrichum gloeosporioides* after 12 days incubation.

Treatment	Mycelial growth (cm)	PIC%	Sporulation
Control	9.00 <sup>a</sup>	-	7.40 <sup>a</sup>
Lemon gras	8.10 <sup>b</sup>	10.37	7.08 <sup>b</sup>
Neem	8.05 <sup>b</sup>	10.66	6.20 <sup>e</sup>
Cinnamon	8.04 <sup>b</sup>	10.83	7.18 <sup>b</sup>
Lemon balm	8.00 <sup>b</sup>	11.78	6.78 <sup>c</sup>
Bitter melon	7.70 <sup>c</sup>	14.89	5.85 <sup>f</sup>
Mint	7.50 <sup>c</sup>	17.20	6.18 <sup>e</sup>
Eucalyptus	7.40 <sup>c</sup>	17.93	6.45 <sup>d</sup>
Ginger	7.20 <sup>c</sup>	20.23	6.13 <sup>e</sup>
Black pepper	7.07 <sup>c</sup>	21.82	6.53 <sup>d</sup>
Garlic	4.50 <sup>d</sup>	50.37	6.90 <sup>c</sup>
CV %	4.91	-	2.24

Means followed by the same letter do not differ significantly by the Scott- Knott grouping test at the level of 5% probability.

**Table 2.** Effect of different plant extracts on spore germination of the fungus *Colletotrichum gloeosporioides* after 12 days incubation.

Treatment	Germinated spores	PIG %
Control	100 <sup>a</sup>	-
Lemon gras	18.72 <sup>g</sup>	81.28
Neem	24.93 <sup>f</sup>	75.07
Cinnamon	74.50 <sup>b</sup>	25.50
Lemon balm	61.70 <sup>c</sup>	38.30
Bitter melon	11.5 <sup>h</sup>	88.5
Mint	17.63 <sup>g</sup>	82.37
Eucalyptus	40.00 <sup>e</sup>	60.00
Ginger	4.68 <sup>i</sup>	95.32
Black pepper	46.4 <sup>d</sup>	53.6
Garlic	8.55 <sup>h</sup>	91.45

Means followed by the same letter do not differ significantly by the Scott- Knott grouping test at the level of 5% probability.

showed potential for spore germination inhibition (Table 2). However, the ginger plant extract presented the highest spore germination inhibition (95.32%), followed by the extracts of garlic (91.45%), bitter melon (88.5%), mint (82.37%) and lemon grass (81.28%). This inhibition was observed by Amadi et al. (2014) when ginger and guava extracts were used on the sporulation and germination of fungus spores stored in melons and was observed that the sporulation and spore germination were inhibited of *Aspergillus flavus*, *A. niger*, *Rhizopus stolonifer* and *Fusarium*.

Only cinnamon and lemon balm, of the plant extracts tested, presented germination inhibition below 50%.

Brito and Nascimento (2015) obtained similar results

when they studied plant extract fungitoxic potential on *Curvularia eragrostidis* and observed that garlic, ginger, neem and citronela, starting at 25% concentration, presented bigger plant toxic effects in the *in vitro* analysis; reducing mycelial growth and sporulation as well as fungus germination. Marcondes et al. (2014) also observed that 20% garlic extract completely inhibited *C. gloeosporioides* conidia germination and reduced the number and germination of *Fusarium moniliforme* conidia.

The fungitoxicity of garlic extracts on fungus spore germination has been reported in several other studies, that it decrease the germination of sexed spores and conidia of a range of fungi pathogenic to plants (Souza

and Soares, 2013; Wilson et al., 1997).

Lorenzi and Matos (2002) reported that aromatic herbs, such as garlic and ginger, have bacteriocide and fungicide action, because they contain allicin, inulin, gingerol and shogaol in their chemical composition that confer high potential to these plants for control of several pathogens. Morais (2004), observed that 20% garlic aqueous extract concentrations inhibited *Fusarium oxysporum* Schlecht., Emend., Snyder., and Hansen. conidia germination.

Oliveira et al. (2008) reported that the use of neem and garlic plant extracts at different concentrations (20, 30 and 40%) may be control alternatives for *Fusarium gutiforme*. Souza et al. (2007) reported that garlic and lemon grass (*C. citratus* Stapf.) extracts inhibited germination of the fungus *Fusarium proliferatum* (Matsush.), but were more efficient at concentrations above 2.5%. According to the authors, these extracts have inhibitory constituents, thus indicating the possibility of using plant extracts to protect the host and/or eradicate the pathogen.

Conidia germination inhibition and deformities in the germination tubes due to plant extract action have been reported. Bonaldo et al. (2004) assessed autoclaved *Corymbia citriodora* aqueous extract on *Colletotrichum lagenarium* (Pass.) Ellis and Halst. conidia germination and observed that at concentrations of over 5% fresh leaves (p/v), there was 90% germination inhibition and when 25% plant extracts were used the inhibition was 100%.

Deformity has also been reported concerning the germination tube morphology of the germinated conidia, an effect accentuated by increase in concentration. Souza et al. (2007) observed decrease in *F. proliferatum* spore germination due to increased concentration in garlic and lemon grass plant extracts; at 10% concentration there was 90 and 81% inhibition, for the two extracts, respectively. Bonaldo et al. (2007) assessed *C. citriodora* essential oil at different doses (5 to 60 µL), and reported 100% inhibition in apressorium germination and formation in *Colletotrichum sublineolum* for all the doses tested.

The variation in the results obtained when using plant extracts for plant disease control was probably due to the variable quantity and chemical composition of the plant extracts (Silva, 2006).

#### **Assessment of plant extract action on anthracnose control in *H. psitacorum* cv Golden Torche and *H. rostrata***

The results of the *in vivo* test with *C. gloesporioides* in *H. psitacorum* cv. Golden torch showed that all the plant extracts tested were efficient in reducing lesion severity on the inflorescences. The garlic, ginger and eucalyptus extracts gave the best results, significantly reducing

lesion diameter (Figure 1).

The first symptoms started to appear three days after inoculation, the final assessment was made seven days after the first symptoms were manifested and was characterised as circular, dark brown necrotic lesions around the inoculation location (Figure 2).

Disease control using plant extracts has been shown in other pathogen systems. Cinnamon essential oil sprayed on papaya plants maintained low lesion percentage on leaves for up to 14 days after inoculation with *Corynespora cassiicola*; but when it was applied after the start of infection, it could not control the disease (Bitu et al., 2016).

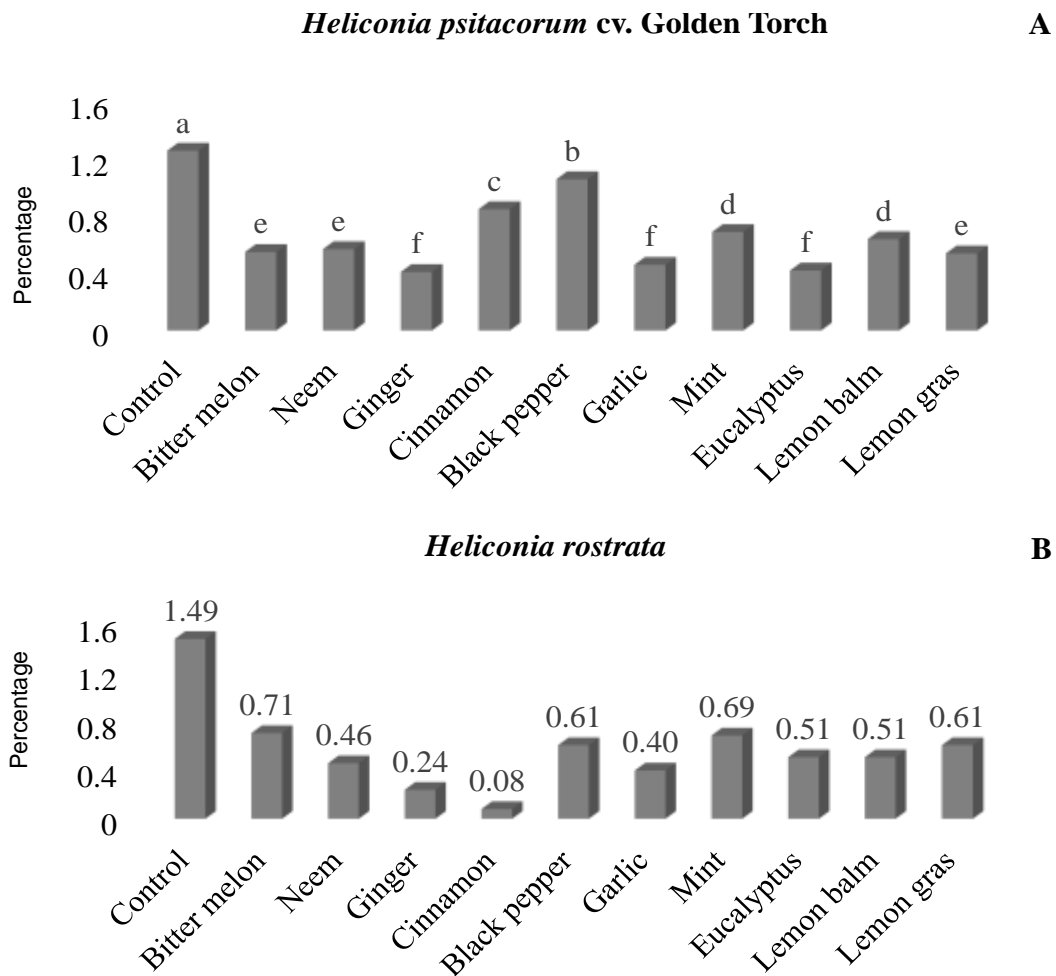
Eucalyptus and citronella essential oils reduced severity of lemon grass rust, but the efficiency of treatments with essential oils was directly related to the environmental conditions and the characteristics of the pathogen system involved (Lorenzetti et al., 2012).

The results of the *in vitro* test with *C. gloesporioides* in *H. rostrata* showed that all the extracts tested were efficient in reducing the lesion severity on the inflorescences (Figure 1), but the cinnamon and ginger extracts gave the best results, significantly reducing the lesion diameter.

Similar results were reported by Itako et al. (2008) who assessed the protective effect of root aqueous extracts (EBAs) of the medicinal plants, *Achillea millefolium* (yarrow), *Artemisia camphorata* (camphor), *C. citratus* (lemon grass) and *Rosmarinus officinalis* (rosemary) against *Alternaria solani* in tomato plants in a greenhouse. A significant reduction was observed in the number of lesions compared to the control and the extracts had a systemic effect.

Other examples have been reported, such as control of brown spot (*Bipolaris sorokiniana*) in wheat, using camphor aqueous extract (*Artemisia camphorata*) (Franzener et al., 2003), tomato plant oidium (*Oidium lycopersici*) by *Azadirachta indica* emulsion oil (Carneiro, 2003), of anthracnose (*C. lagenarium*) in cucumber by *C. citriodora* extract (Bonaldo et al., 2004) and white mold (*Sclerotinia sclerotiorum*) in lettuce by *Z. officinale* (Rodrigues et al., 2007); indicating that plant extracts and vegetable oils are promising alternatives for use in plant disease control.

Plant extracts produce biologically active substances, that influence the metabolism of a determined organism and they act by contact or systemically triggering metabolic pathways. According to Schwan-Estrada and Stangarlin (2005) root plant extracts and/or essential oil actions include direct anti-fungal action, physiological alterations in the plant, or by inducing enzymes related to the pathogenesis, phytoalexins, and leaf lignification. Therefore, fractioning the metabolites of these plants and determining the biological activity of these molecules in relation to the elicitory or antimicrobial activity may contribute to greater understanding that reinforces their possible use as an alternative method for plant disease



**Figure 1.** Effect of the plant extracts on reducing the severity of leaf spots caused by *C. gloesporioides* in inflorescences of *Heliconia psitacorum* cv. Golden Torch (A) and *Heliconia rostrata* (B), 10 days after inoculation. Means followed by the same letter do not differ significantly by the Scott- Knott grouping test at the level of 5% probability.



**Figure 2.** Inflorescences of *Heliconia psitacorum* cv. Golden Torche (A) and *Heliconia rostrata* (B) with anthracnose symptoms.



control (Schwan-Estrada et al., 2000).

## Conclusion

All the extracts presented anti-fungal activity, in greater or lesser intensity, compared to the control. The garlic extract resulted in the highest mycelial growth inhibition rate. The bitter melon, ginger, mint, popcorn eucalyptus and bitter melon extracts were more efficacious, interfering in spore formation; while the ginger extract most reduced spore germination inhibiting germination tube emission. It was observed that all the plant extracts tested were efficient in reducing lesion severity in the inflorescence, showing that the use of plant extracts may be a promising alternative for managing the diseases that affect helconia post-harvest.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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