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Full Length Research Paper

Changes in some behavioral, hematological and biochemical indices of air-breathing *Clarias gariepinus* [BUCHELL, 1822] exposed to pharmaceutical effluent

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This work aimed at evaluating the behavioral, biochemical and hematological effects of pharmaceutical effluent in laboratory population of *Clarias gariepinus* using a static renewal bioassay system. Fish specimens were collected and exposed to five (0.04, 0.06, 0.08, 0.10 and 0.12 mgL^{-1}) sublethal concentrations of the effluent including a control experiment. Different dose dependent behavioral responses such as erratic swimming, gasp for breath, restlessness, and constant upward movement were observed in exposed fish. There was a steady decrease in the value of red blood cell (RBC), hemoglobin (Hb) and packed cell volume (PCV) as concentrations increase compared to the control stock. White blood cell count was found to be significantly ($p<0.05$) higher as the concentration of the test medium increases. Irregular level of lymphocytes and granulocytes across all concentrations was observed and levels of lymphocytes and granulocytes were significantly ($p<0.05$) increased in all effluent-treated fish samples during the exposure period. Different levels of total protein, glucose, cholesterol, etc., were obtained in the control and effluent-treated fish samples ($p<0.05$). The levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (serum enzymes) and lactate dehydrogenase were significantly ($p<0.05$) higher in the exposed *C. gariepinus*. The hematological and biochemical alterations in the effluent treated *C. gariepinus*, which were strongly indicative of cellular damages. This might be attributed to toxic effects of the phamarceutical effluent. Consequently, direct discharged of untreated or partially treated pharmaceutical effluent should be discouraged as this calls for public health concern.

Key words: Hematology, biochemical, pharmaceutical, *Clarias gariepinus*, effluent.

INTRODUCTION

Fishes live intimately with their environment and are exposed to varied degree of physical and chemical change in the aquatic phase and such changes can alter their general physiology and metabolism, which may be evaluated using their haematological and biochemical indices. The use of haematological indices in assessment of fish physiological has been used as an index of fish health status. These haematological indices are used as a tool to detect physiological changes, as a result of exposure to different environmental stressors such as

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handling, pollutants, metals, hypoxia, anesthetics, season and acclimation (Ogundiran et al., 2007; Ayandiran et al., 2010).

Pharmaceutical compounds are used for several beneficial purposes in modern society but simultaneously pharma industries are releasing very toxic contaminants directly or after chemical modifications into the environment (Halling-Sorenson et al., 2005). Moreover, pharmaceutical waste compounds may enter the environment by different routes such as discharge of treated wastewater, seepage from landfills sites, sewer lines, runoff from animal wastes, etc. (Glassmeyer et al., 2005). Various physical and biological processes occurring in aquatic ecosystem may cause reduction of many pharmaceutical compounds, trace concentrations of human and veterinary pharmaceutical compounds as well as their metabolites have been detected in different water bodies like surface water, groundwater and drinking water sources (Bruce et al., 2010; Benotti et al., 2009).

Different industries including pharmaceuticals, chemicals, paints, etc., are speedily growing in Nigeria, which dispose off their effluents into the streams either directly or after partial treatment. Pharmaceutical compounds had been established to reach the environment and can be considered as environmental pollutants. Several pharmaceutical production facilities were found to be sources of much higher environmental pollutants concentration than those resulting from the applications of drugs (Larsson et al., 2007). Generally, pharmaceutical industries generate a huge quantity of wastes during manufacturing and maintenance operations and trace amount of this wastewater for longer duration in the environment may cause considerable adverse effects to human health and aquatic life (Benotti et al., 2009).

Information about the phamarceutical effluent toxicity at ecosystem level is limited and there is a need to investigate the toxicity impact of this effluent on fish. Therefore, this present study aimed at investigating the impact of sublethal concentrations of pharmaceutical effluent on catfish (*Clarias gariepinus*) with special reference to the haematological and biochemical changes.

MATERIALS AND METHODS

Experimental fish and chemicals

A total of 350 freshwater Juvenile African catfish [*C. gariepinus*] were procured from a local fish farm in Ogbomoso, Nigeria and were transported to the fisheries laboratory of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso. The fish specimens were given prophylactic treatment immediately by bathing them repeatedly in 0.05% $KMNO_4$ to prevent possibility of any dermal infection. The fish stock was acclimated to the laboratory condition in six [600-L] plastic tank capacities for four weeks using non-chlorinated bore-hole water fetched from the running taps of the university. They were fed with 35% protein diet at 3% body weight daily in the early hour of the day. In order to avoid oxygen depletion, the holding tanks were continuously aerated using air pumps. The experiment was carried out indoor to maintain suitable photoperiod. The fish were treated in accordance with the rule conforming to worldwide conventional principles of laboratory animal care. The behavioral responses of the fish held in both experimental and control conditions were monitored on hourly basis. The physico-chemical analysis of the test medium was analyzed daily using standard methods of APHA (2012). Pharmaceutical effluents used were collected early in the morning at the discharge point between 7 and 8 AM from Sofak Pharmaceutical Company, Ogbomoso. The used test media from the pharmaceutical effluent was prepared by diluting the stock solution with a constant factor covering a large range.

Definitive experiment

Three hundred fish from the acclimatized batch were used for the definitive experiment. The fish were not fed 24 h prior to commencement of the exposure to the test media throughout the duration of the experiment, as recommended by Ward and Parrish (1982) and Reish and Oshida (1987). The fish were randomly selected into six groups containing 50 fish each, regardless of sex. Each group was further randomized into two replicates, with 25 fish per replicate in 100-L capacity glass aquaria. Fish in the first group were exposed to borehole water only [control] and fish in the second group were exposed to 0.04 mg/L of the test media, respectively. The third, fourth, fifth and sixth groups were treated with 0.06, 0.08, 0.10 and 0.12 mg/L of the test media, respectively. The experiment was conducted within six weeks in a static renewal bioassay system in which the water and the effluent were changed daily to maintain constant effluent concentration. The test concentrations of the pharmaceutical effluent were prepared by dilution from the stock. The dose schedule selected was based on presumptive investigation involving a range finding test from previous reports in literatures.

Collection of blood sample and hematological analysis

At the expiration of the experiment, blood needed for the haematological investigation was collected from anesthetized fish samples in each treatment and control stocks with MS 222 [Ethyl 3-aminobenzoate methane sulfonate salt] to minimize stress. Blood was obtained by cardiac puncture using a hypodermic heparinized syringe. The collected blood samples were transferred into small vials, which were also previously rinsed with heparin. Red blood cell (RBC) counts were estimated using a Neubauer hemocytometer, as described by Rusia et al. (1992) and Allen (1993). 0.02 mL of blood was pipetted from the blood sample and added to 4 mL of the RBC diluting fluid [Toisson's solution], in a clean test tube to make a 1:20 dilution of the blood sample (Clara et al., 2004). The diluted blood sample was loaded onto a Neubauer counting chamber, and all RBCs in the five groups of 16 small squares in the central area of the Neubauer chamber was counted using a light microscope at 40X objective. The number of cells counted for each sample was multiplied by 10000 to obtain the RBC count per microliter of blood. Hematocrit [packed cell volume (PCV)] was determined using the microhematocrit method of Nelson and Morris (1989), in which the capillary tubes were filled with blood and centrifuged for 5 min at 14000× *g* using a microhematocrit centrifuge (Hawkesley & Sons, Ltd, Lancing, UK) at room temperature. Soon after centrifuging, the hematocrit was read using the microhematocrit reader.

The result was expressed as the percentage of whole blood. Hemoglobin determination was done using the cyanmethemoglobin method (Blaxhall and Daisley, 1973). About 0.02 ml of blood was

Table 1. Physico-chemical characteristics of the pharmaceutical effluent used.

Parameter	Effluent value	Standard value
Dissolved Oxygen (mg/L)	0.83±0.03	6.0
pH	5.70±0.01	6.5-9.5
Total Solids (mg/L)	318.67±8.82	<1000
BOD (mg/L)	127.00±1.53	50
COD (mg/L)	241.33±9,91	ND
Alkalinity	3.83±0.08	ND
Total Hardness	72.87±0.81	<200
Conductivity (µS)	979.67±0.88	NP
SO_4^- (mg/L)	18.17±0.12	500
Cl^- (mg/L)	34.67±0.11	ND
PO_4^- (mg/L)	16.50±0.17	5.0
Lead (mg/L)	0.05±0.02	<0.01
Copper (mg/L)	0.06±0.01	<2.0
Zinc (mg/L)	0.04±1.01	<1.0
Iron (mg/L)	0.08±0.02	<1.0

ND, Not determined.

mixed with 4 mL of Drabkin's solution and this was allowed to stand for 10 min to attain full color development. Absorbance was read at 540 nm with a Unican spectrophotometer against the blank. For determination of leucocytes, 0.02 mL of blood was pipetted into a small test tube containing 0.38 mL of white blood cell (WBC) diluting fluid (Turk's solution) to make a 1:20 dilution of the blood sample. The diluted sample was loaded onto the Neubauer counting chamber, and all cells on the four corner squares were counted using a light microscope at 10X objective. The total number of WBCs was calculated in $mm^3 \times 10^4$. Aspartate aminotransferase (AST) and alanine aminotranferease (ALT) were analyzed according to the method of Reitman and Frankel (1957), alkaline phosphatase (ALP) according to Bessey et al. (1946) and lactate dehydrogenase (LDH), according to the method of Wahua (1999).

Statistical analysis

The data obtained from the results of all the analysis were subjected to statistical analysis using SPSS 16.0 software. Data were subjected to one-way analysis of variance, Duncan multiple range test was used to determine the significant difference at the 5% probability level, and the results were expressed as the means standard error.

RESULTS AND DISCUSSION

Physico-chemical analysis of the test medium

The physico-chemical characteristics of the effluent used is shown in Table 1 and virtually all the analyzed parameters were found to exceed (WHO, 2011) standards for effluent discharge into any category of water bodies. The results of the hematological and biochemical responses of the test organism to varying concentrations of pharmaceutical effluent are show in Tables 2 and 3, respectively, while the activities of some selected serum enzymes are shown in Table 4. Most of the physico-chemical attributes of the effluent used showed deviations from the WHO (2011) of the permissible level of physical, chemical and heavy metals in any categories of water. Thus, continuous discharge of the effluent into water bodies over time might lead into the bioaccumulation of metals in fish tissues and organisms in nearby water bodies.

Behavioral responses

Exposure of the test organism *C. gariepinus* to pharmaceutical effluent caused visible behavioural changes in the fish. After 60 min, their swimming activity slowed down, the test fishes felt suffocation, they tried to stay at upper water surface to gasp for air, irregular and jerky movement, and loss of body equilibrium were also pronounced. Also, they settle down at the bottom of the aquaria and those that are not able to withstand the situation died. Fishes of control group were free from such behavioural changes. This conforms to the submission of Adewoye (2010).

Hematological and biochemical response

Fishes are in direct contact with their surrounding environment and any change in the environment will be reflected as changes in their physiological processes and survival. Fishes possess shorter development time compared to mammalian species. The present study revealed an interesting pattern of response of the haematological variables in effluent exposed fish. In

Table 2. Hematological indices of *Clarias gariepinus* exposed to pharmaceutical effluent.

Parameter	0.00 (mg/L)	0.04 (mg/L)	0.06 (mg/L)	0.08 (mg/L)	0.10 (mg/L)	0.12 (mg/L)
Packed cell volume (%)	26.00±2.00	24.5±0.5	24.00±1.00	22.00±2.00	23.00±1.00	21.00±1.00
Red blood cell (µl)	3.10±0.10	2.95±0.25	2.85±0.05	3.15±0.05	1.10±0.10	1.00±0.10
White blood cell (µl)	1270±0.10	2200±0.35	3000±0.05	9200±0.01	10000±0.10	8700±0.10
Hemoglobin (g/dl)	7.29±0.65	5.21±2.22	5.01±0.01	4.75±1.10	4.33±1.11	4.01±1.19
Granulocyte (%)	37.00±2.01	27.50±2.50	30.5±2.50	32.00±2.00	28.50±0.50	27.50±1.50
Leucocyte (%)	70.00±2.11	72.50±2.50	69.50±2.50	58.00±2.00	71.51±0.55	72.50±1.35

Table 3. Biochemical indices of *Clarias gariepinus* exposed to pharmaceutical effluent.

Parameter (mg/L)	Total protein	Glucose	Cholesterol	Albumin	Chloride	Potassium	Calcium
Control	35.00±3.00	86.10±4.00	100.0±2.21	16.00±0.01	94.50±2.50	3.60±0.02	2.35±0.05
0.04	32.51±1.50	79.00±1.00	108.0±2.01	19.50±1.50	91.00±0.01	3.40±0.20	2.10±0.20
0.06	31.00±3.00	73.00±1.00	115.0±0.09	19.00±1.00	88.50±1.50	3.35±0.05	2.00±0.15
0.08	33.00±2.00	74.00±0.01	112.0±0.04	23.50±0.05	89.50±0.01	3.15±0.05	2.21±0.02
0.10	27.00±1.00	61.11±2.00	126.1±5.50	21.55±1.45	77.91±2.11	3.11±0.01	1.21±0.01
0.12	25.15±2.31	55.01±2.22	119.0±13.29	27.01±1.56	75.55±1.00	3.00±0.07	1.11±0.02

Table 4. Response of serum enzyme of *Clarias gariepinus* to varying concentrations of pharmaceutical effluent.

Parameter (µkat^{1})	0.00 (mg/L)	0.04 (mg/L)	0.06 (mg/L)	0.08 (mg/L)	0.10 (mg/L)	0.12 (mg/L)
Alanine aminotransferase	0.45±0.10	0.39±0.51	0.33±0.01	0.42±0.11	0.46±0.05	0.49±1.09
Aspartate aminotransferase	3.99±0.11	2.12±0.15	2.21±0.55	2.01±1.15	2.00±0.00	1.74±1.13
Alkaline phosphate	1270±0.10	2200±0.35	3000±0.05	9200±0.01	10000±0.10	8700±0.10
Lactate dehydrogenase	15.21±1.23	11.39±2.05	12.46±0.19	10.25±1.21	8.20±2.00	9.22±4.65

addition, different concentrations resulted in an anemic condition in fish, as shown from decreased RBC, PCV and hemoglobin (Hb) values as effluent concentration increases. From the present results, reduction in RBC count, PCV and Hb concentrations of tested fish compared to control may be due partly to the presence of heavy metals and other pollutants in the utilized effluent and the toxicity of this effluent may cause RBC lysis. Previous studies had shown a decrease in RBC counts, relative PCV and Hb values when fish were exposed to cassava effluent (Adekunle et al., 2007), diazinon (Svoboda et al., 2001), textile dyes (Al-sabti, 2000) and soap and detergent effluent (Ogundiran et al., 2007). These findings are in agreement with the present results. On the contrary, an increase in RBC, Hb and Ht values were recorded in African catfish on exposure to Gold crew (Alagoaa et al., 2009), to copper (Mazon et al., 2002) and in Nile Tilapia and catfish exposed to lead (Al-Akela et al., 2000). Deformed RBCs were detected on exposure to cadmium (Witeska et al., 2006) and environmental pollution (Pacheco and Santos 2002). Moreover, marked reduction in the value of hematological indices as documented in this work may also be attributed to a reduction in the level of cellular iron resulting in reduced oxygen carrying capacities of the blood which eventually stimulates erythrocytic degradation.

The concentration of hemoglobin decreased significantly ($p< 0.05$) in the blood of fish exposed to the pharmaceutical effluent. Heavy metals have been reported to alter the properties of haemoglobin by decreasing their affinity towards oxygen binding capacity rendering the erythrocytes more fragile and permeable (Witeska et al., 2006; Ogundiran et al., 2007; Vinodhini and Narayanan 2009) that probably results in cell swelling deformation and damage observed. It is evidently shown that cadmium influences the differential blood count (Gill et al., 1993). The results are in good concurrence with earlier works (Vinodhini and Narayanan 2009; Vutkuru 2005) that reported a significant decrease in RBC's hemoglobin and packed cell volume of fresh water fish exposed to heavy metals. The perturbation in these blood indices may be attributed to a defense reaction against toxicity through the stimulation of erythropoiesis. The

related decreases in hematological indices implicate the toxic effect of the pharmaceutical wastewater that affects both metabolic and hematopoietic activities of *C. gariepinus*. Exposure to high concentration of the toxicant affected feeding behavior of *C. gariepinus*, with fish exposed to 14.1589 m/L effluent consuming less than 80.0% of the food supplied. And consequently, the observed dose-based reduction in haematological parameters in *C. gariepinus* in this study therefore conforms to the report of Tacon (1993) and Osuigwe et al., (2005) that nutritionally deficient diets cause decrease in haemaglobin concentration, reduced haematocrit and red blood cell count. Physiologically, haemoglobin is crucial to the survival of fish, being directly related to the oxygen binding capacity of blood. However, the reduction observed in this study may not have had a deleterious/lethal effect on *C. gariepinus,* given that the values are within the normal range recorded for African catfish (Musa and Omoregie 1999; Ayandiran et al., 2010; Yekeen and Fawole 2011). The present haematological data provides valuable information in assessing the health of *C. gariepinus* and in monitoring stress responses to the metal wastewater.

Glucose and total protein concentrations in blood of the *C. gariepinus* exposed to different sublethal concentrations of pharmaceutical effluent showed that there was a significant decrease as the concentration of the effluent increases (Table 2). The glucose concentration in animals exposed to sub-lethal concentrations of pharmaceutical effluent was significantly different ($p<0.05$) from the concentrations in control animals. Albumin concentrations in blood of the *C. gariepinus* exposed-fish showed that there was a slight significant ($p<0.05$) increase in the concentrations in the exposed animals as compared to the controls. The blood electrolyte [Sodium, Potassium and Calcium] recorded a significant ($p<0.05$) decrease in their respective values as the concentration of the effluent increases. All the anomalies recorded in the biochemical response of pharmaceutical effluent-exposed *C. gariepinus* may be stress due to the potency of the effluent-used.

The activities of serum enzymes (AST, ALT, ALP and LDH) were significantly ($p<0.05$) higher in the exposed *C. gariepinus* compared to the control. The significant ($p<0.05$) decrease in the activities of serum enzymes [AST, ALT and ALP] in the exposed fish compared to the control stock may have resulted from cellular damage in these fish, which might have arisen from the potency of the pharmaceutical effluent. This observation was similar to what Ozgur et al., (2011) reported where they observed significant increase ($p<0.05$) in serum enzyme activities of Nile tilapia *Oreochromis niloticus* exposed to a pesticide cypermetrin and two metals copper and lead. Serum activities also increased significantly in African catfish when Nkpondion et al. (2016) exposed the fishes to detergent. This is because serum enzymes are cytoplasmic in nature and are only released into blood circulation after cellular damage (Nkpondion et al., 2016).

Conclusions

The effluent-water levels had negative direct or indirect effects on the haematological and biochemical parameters of *C. gariepinus*. The blood parameters of *C. gariepinus* not only revealed cellular disturbances but also adaptive responses. Increase in leukocytes implies a mobilization of cell defense, although the reduction of the small lymphocytes percentage suggests a secondary effect of effluent. On the other hand, the decrease in red blood cell parameters [RBC, Hb and PCV] indicates an aneamic condition which gradually progresses upon prolonged exposure eventually cause hypochromic macrocytic anemia attributed to the swelling of the red blood cells, haemodilution and impaired haemoglobin synthesis. The increase in developing haemocytoplast and myelocytes emphasizes the compensatory and defensive reaction of fish to pollution. Contamination of aquatic environment by heavy metals whether as a consequence of acute or chronic events constitutes additional source of stress for aquatic organisms. Toxicants and pollutants can result in several physiological dysfunctions in fish which could induce changes in blood parameters. Therefore, the reduction and increase in these blood parameters are indication of hyperglycaemia and hypoproteinemia caused by exposure to the phostoxin concentrations. Generally, the effects of the pharmaceutical effluent were more pronounced in serum enzymes activities than in the haematological responses of the exposed *C. gariepinus*. However, significant ($p<0.05$) increase in serum enzymes and WBC counts of the wild *C. gariepinus* were indicative of cellular damages in exposed fishes. Therefore, indiscriminate consumption of fish most especially from a polluted environment should be discouraged.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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An efficient *in-vitro* regeneration system of Taro (*Colocasia esculenta* L. Schott) using apical meristems

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Taro (*Colocasia esculenta* L. Schott) is an important crop for food and nutrition security, incomes, and livelihood of people in developing countries. Its cultivation and productivity have been limited mainly due to a lack of quality planting materials. Therefore, this study developed an effective micropropagation method for two common taro varieties in Kenya (Purple Wild and Dasheen) using apical meristem derived from portions of the corm and base of leaf petioles. This study optimized the 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) levels for a better shoot and root development. The highest shoot induction was observed in both varieties when Murashige & Skoog (MS) media was supplemented with 2 mg/L of BAP. Similarly, the maximum rooting response was achieved in both varieties on half-strength MS media supplemented with 0.5 mg/L of IBA. The study also revealed significant interactions between variety × BAP levels and variety × IBA levels (P<0.0001) for shoot and root development, respectively. This apical meristem-based micropropagation method developed in this study can be used for rapid multiplication of genetically clean planting materials for commercial uses and to establish taro transformation protocols.

Key words: Taro, 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), shoot induction, root induction, regeneration.

INTRODUCTION

Taro (*Colocasia esculenta* L. Schott) is one of the edible members of the family Araceae distributed across the world, particularly in the tropics (Okonkwo, 1993). It is native to Southeast Asia (Matthews, 1991; Yoshino, 2002) and widely cultivated as a staple food crop in the Pacific Islands, Asia, and Africa (Onwueme, 1999). The global acreage under taro production for 2019 was 1.957 million hectares, with a total production of 10.54 million

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tons. Nigeria, China, Cameroon, Ghana, and Papua New Guinea were the top five producers of taro in the world (FAO, 2021). Taro is mainly cultivated for corm production, but the pseudo-stems and leaves are also edible and used to prepare various traditional food dishes. Taro corms are a good source of digestible starch, crude fiber, minerals, vitamin C, thiamin, riboflavin, and niacin (Boampong et al., 2019; Jane et al., 1992; Kidanemariam, 2018). Both corms and leaves are rich in crude proteins (Temesgen and Retta, 2015). Besides nutritional values, taro is an important food security crop in developing countries because of its high yield potential, that is, as high as 110 t/ha (Lebot, 2009). Although taro is primarily grown for domestic consumption, the production of taro for cash generation has been a rewarding business. Moreover, taro is adapted to a wide range of environments and agricultural practices (Kantaka, 2004), making taro a crop of choice under changing climatic conditions.

Despite the importance of taro in food and nutrition security, incomes, and livelihood of rural people, the current global productivity of taro is estimated around 5.39 t/ha, which is about 5% of its experimental yield (Lebot, 2009). Low productivity and limited cultivation of taro are attributed to several factors, including a high incidence of pest and diseases; scarcity of quality planting materials; labour-intensive traditional production system; difficulties in post-harvest handling and marketing; and low investment in taro research and extension (Onwueme, 1999). Taro is commonly propagated through vegetative means like side suckers, small corms, and corm pieces. These propagation materials are bulky in nature and low throughput, and the availability of these planting materials is seasonal. Moreover, these vegetative planting materials often serve as vehicles for various pests and diseases from one taro crop to the next crop and subsequently compromise the quality and quantity of taro production. Therefore, the availability of quality planting material in sufficient quantities has been a major challenge among taro producers. More importantly, the taro seeds system lacks an efficient and high throughput micropropagation system that helps in the mass propagation of quality planting materials.

The tissue culture or micropropagation technique provides a sustainable solution to the problems associated with conventional propagation by enabling rapid production of high-quality, disease-free, and uniform planting materials (IAEA, 2004). Since tissue culture is performed in a controlled laboratory environment, the multiplication of planting materials could be achieved all year-round. The tissue culture technique has been successfully applied on thousands of plant species (Fay, 1992; Villalobos and Engelmann, 1995; Jackson et al., 2001; Sarasan et al., 2006), but the use of this technique for *in-vitro* regeneration and mass production of taro planting material has been limited in Kenya primarily due to the lack of efficient regeneration and mass multiplication protocols. Several protocols for taro tissue culture are available (Yam et al., 1990; Tuia, 1997; Minas, 2002; Hossain, 2012), including the one that uses locally available nutrients to substitute MS medium (Ngetich et al., 2015). All these efforts to develop taro micropropagation protocols could be due to genotype dependent response of taro to micropropagation methods, variations in growth media and culture conditions, types of explants used in micropropagation, and to reduce the costs.

The direct and indirect shoot organogenesis are considered the best micropropagation method due to a low cost, minimal soma-clonal variations, and high throughput production system (Mukami et al., 2018; Burner and Grisham, 1995). Apical meristem culture eliminates viruses in many plant species, thus helps in the production of disease-free planting materials and achieving better yields than conventional planting materials (Wang and Valkonen, 2008, 2009). In this study, organogenesis-based regeneration protocols was developed for two common taro varieties of Kenya, that is, Purple Wild and Dasheen, through optimizing cytokinin and auxin levels for the shoot and root regeneration, respectively.

MATERIALS AND METHODS

Collection and management of planting materials

Two taro varieties, Purple Wild and Dasheen were collected from Busia (0.4347 °N, 34.2422 °E), Kakamega (0.2827 °N, 34.7519 °E), Kiambu (1.0314 °S, 36.8681 °E), Kisii (0.8067 °S, 34.7741 °E), Machakos (1.5177 °S, 37.2634 °E), Meru (0.3557 °N, 37.8088 °E), Murang'a (0.7839 °S, 37.0400 °E), Nyeri (0.4197 °S, 37.0400 °E), and Siaya (0.0617 °S, 34.2422 °E) counties of Kenya in August 2017. Taro plantlets were collected in polythene bags, labelled, and transported to the greenhouse of International Livestock Research Institute, Nairobi, Kenya. The taro plantlets were established in 4 L pots (Planter 1 H200 × Dia268 mm) with autoclaved soil consisting of forest soil and manure in the ratio of 2:1 by volume. Notably, a hand full of gravel was dispensed at the bottom of each for drainage of excess water. These planets were maintained in a screen house at ambient conditions for four months before using these stock plants in subsequent experiments.

Media and explants preparation

Murashige and Skoog (MS) media was prepared using 4.4 g/L of MS media with basal salts, 30 g/L of sucrose dissolved in distilled water, and pH was adjusted to 5.8 using 0.1 N HCl and 0.1 N NaOH, and 3 g of Gelrite added to the bottle. All the media, glassware, and metallic equipment, e.g., forceps and blade holders used in experiments, were autoclaved at 121°C at 15 psi for 20 min. The explants comprising the corm top attached to the base of the petioles were harvested from stock plants in the greenhouse, trimmed to a length of 2 cm, and taken to the laboratory. The explants were thoroughly washed using running tap water for 30 min and immersed in a 1 L glass beaker filled with tap water containing soap antibacterial (Dettol) and two drops (100 µl/L) of tween 20 and swirled gently by hand for 30 min. The explants were

Table 1. Responses of taro varieties (Dasheen and Purple Wild) to five different concentrations of Benzyl-aminopurine (BAP) for shoot induction (at 15 days) and shoot multiplication (30 days).

Genotype	BAP concentration (mg/l)	Shoot induction (15 days)	Shoot multiplication (30 days)
Dasheen	0.0	0.033± 0.126^{d}	0.500± 0.205^{f}
	0.5	0.911± 0.126^{c}	3.189± 0.205^{d}
	1.0	1.856± 0.126^{b}	5.211± 0.205^{bc}
	2.0	3.256± 0.126^{a}	7.611± 0.205^{a}
	3.0	1.867± 0.126^{b}	4.911± 0.205^{c}
Purple Wild	0.0	0.189± 0.126^{d}	1.311± 0.205^{e}
	0.5	1.811± 0.126^{b}	4.800± 0.205^{c}
	1.0	2.178± 0.126^{b}	5.544± 0.205^{b}
	2.0	3.033± 0.126^{a}	7.444± 0.205^{a}
	3.0	1.878± 0.126^{b}	4.722± 0.205^{c}
Mean	-	1.701	4.524
Pr > F	-	< 0.0001	< 0.0001

Means (± SE) followed by different alphabets in each column are significantly different ($p \leq 0.0001$) using Duncan multiple range test.

rinsed twice, then transferred to another beaker containing 3 g/L Redomil fungicide (Metalaxyl 8% + Mancozeb 64% WP) an hour with gentle swirling at intervals, and then rinsed thrice using autoclaved-distilled water. After that, the explants were immersed in 70% ethanol for 1 min and rinsed thrice using autoclaved distilled water in a laminar flow chamber. The explants were subjected to 40% JIK regular bleach (added with 100 µl tween-20 L^{-1}) for 20 min, rinsed thrice in autoclaved distilled water, and trimmed to a length of about 1 cm in a laminar flow chamber.

Shoot induction, multiplication, and elongation

The trimmed explants were transferred to shoot induction medium (SIM) comprising MS basal supplemented with 30 g/L sucrose and five 6-benzyl aminopurine (BAP) hormone levels (0, 0.5, 1.0, 2.0, and 3.0 mg/L). For each variety, ten explants were subjected to each BAP level and replicated three times, and the experiment was arranged in a completely randomized design. The cultured explants were maintained in the growth room with the set temperature at 24 ± 2°C for 16/8 h light/dark cycle for 30 days. The induced shoots at 15 days were transferred to fresh SIM and grown for a further 15 days at similar growth conditions to enhance shoot elongation and multiplication. Data on shoot numbers were recorded at fortnightly intervals.

Root induction

The elongated shoots of ~7 cm length generated in the SIM comprising MS basal supplemented with 30 g/L sucrose were transferred to rooting media consisting of MS basal salt supplemented with 30 mg/L sucrose and five IBA concentrations (0, 0.25, 0.5, 0.75, and 1 mg/L). For each variety five explants were subjected to each IBA level and replicated three times, and this experiment was arranged in a completely randomized design. The plantlets were then incubated in the growth room at 24±2°C under 16/8 h light/dark cycle to induce rooting. Data on the total number of roots initiated per shoot and their length was recorded after 25 days.

Hardening and acclimatization

The rooted plantlets were removed from the growth room, immersed in distilled water to remove media, and transferred individually to a disposable plastic cup that acted as a potting container. The container was first filled with gravel and then topped with forest soil and manure in the ratio of 1:2:1 by volume. The plants were labeled according to the variety and the concentration of BAP/IBA. They were grown for 25 days, transferred to the glasshouse, and placed under an improvised humidity chamber for eight days, after which they were removed and exposed to ambient temperature conditions in the greenhouse. The plants were watered daily, and plant survival rates were recorded for each treatment.

Data collection and data analysis

Data on shoots and roots development was recorded after 15 days. The number of induced roots and their length after culturing them for 15 days on the rooting medium was recorded. The percentage survival of the plantlets transferred for acclimatization was recorded after 15 days. Data were subjected to analysis of variance (ANOVA) and treatment means were compared using Duncan Multiple Range Test (DMRT) using the XLSTAT 2020 software (Addinsoft, 2021), and the graphs were plotted using R statistical software (Wickham, 2016).

RESULTS

Effect of BAP concentrations on shooting

The effect of different BAP concentrations on shoot induction and shoot multiplication in two taro varieties are shown in Table 1. The BAP concentrations evaluated in this study were significantly different for shoot induction and multiplication in two taro varieties ($P<0.001$). At 15

Figure 1. Direct organogenesis (A) 15-day old apical meristem initiated on MS medium containing 2 mg/l BAP hormone; (B) 15-day old sub-cultured apical meristem shoots on MS medium containing 2mg/l BAP hormone; (C) Multiple shoots formed at 30 days; (D) Root development in MS medium containing IBA hormone for Purple wild and Dasheen varieties, respectively; (E) Hardened plantlets in plastic cups at 25 days in the glasshouse.

days, the number of shoot induced was the highest at a BAP concentration of 2.0 mg/L for both varieties ($P < 0.001$), but varieties were indifferent for shoot induction in this concentration. The same concentration was the best performer for shoot multiplication in both genotypes, and two varieties were similar for shoot multiplication. When the BAP concentration was 3.0 mg/L, there was a significant decrease in shoot induction and shoot multiplication in both varieties ($P < 0.0001$). A significant interaction was also noted between lower BAP concentration (0.5 mg/l) and varieties e.g., Dasheen had significantly less shoot induction and shoot multiplication than Purple Wild. A schematic of organogenesis in taro in this study is presented in Figure 1.

Effect of IBA concentration rooting

The effect of different IBA concentrations on number of roots and root length in two taro varieties are presented in Figure 2. The different IBA concentrations evaluated in this study deferred significantly for number of root production and root length ($P < 0.0001$). The IBA concentration of 0.5 mg/L induced the highest number of roots in both Dasheen and Purple Wild varieties compared to other treatments and number of root induction in both varieties were similar for this concentration. A gradual decline was observed in the number of root inductions in both taro varieties when IBA concentration was increased to 0.75 and 1 mg/L. Dasheen performed better than Purple Wild for root induction in all levels of IBA concentrations and control treatment, indicating significant interactions among treatments evaluated in this study for root induction ($P<0.0001$).

DISCUSSION

Micropropagation is the *in-vitro* clonal propagation of plants (Bhojwani and Dantu, 2013). Since food insecurity is still a serious global challenge, it is imperative todevelop a direct shoot organogenesis protocol for crops of economic importance as these protocols enable us to produce large numbers of new high-quality plantlets in a relatively short time and space. Micropropagation is a

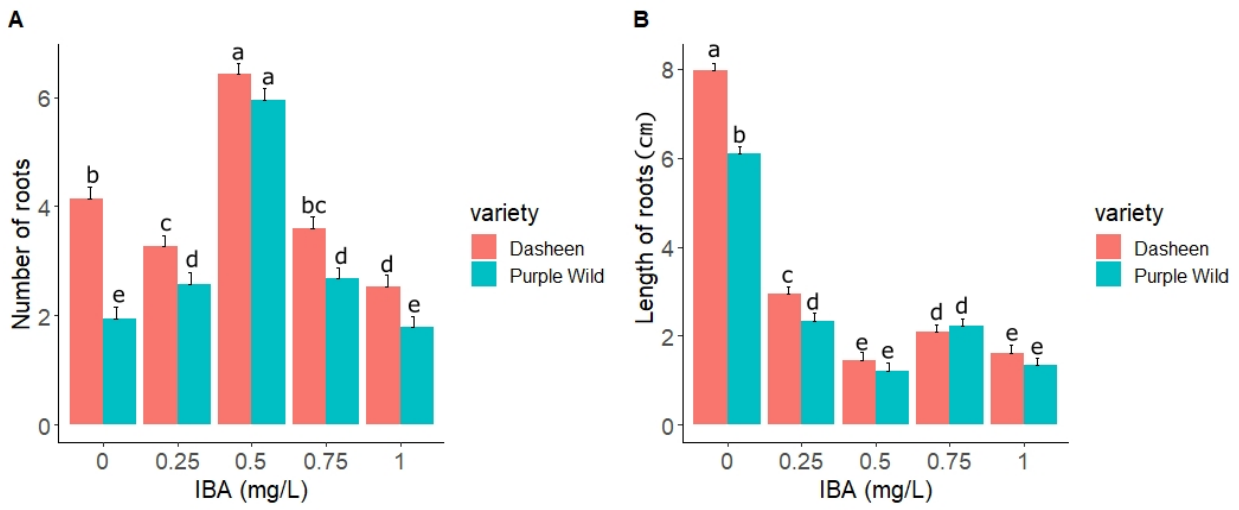

Figure 2. The effect of different IBA concentrations on the number of roots induced in Dasheen and Purple Wild taro varieties at 25 days of culture (A), and the effect of different IBA concentrations on the root length (cm) induced in Dasheen and Purple Wild taro varieties (B). Means were compared using Duncan multiple range test and bars with different letters in a figure are significantly different (p≤ 0.0001).

low-cost technology, and plantlet derived from micropropagation helps in plants germplasm conservation (Gupta et al., 2020). In this study, micropropagation method was developed for two common varieties of taro in Kenya, that is, Dasheen and Purple Wild.

Benzylaminopurine (BAP), an adenine derivative, is an important cytokinin used in shoot induction (Chand et al., 1999). BAP hormone is mainly preferred for *in-vitro* regeneration of monocots (Ramakrishnan et al., 2014). Results generated from shoot proliferation media showed a significant difference in the number of shoots induction between the two taro varieties. Reports by Toledo et al. (1998) state that various varieties of potatoes respond to shoot induction differently because of different genetic background. In this study, shoots and leaves were best produced on the two varieties when cultured on a BAP level of 2 mg/L. Here, we noted that this regime gave the best shoot induction in both Dasheen and Purple Wild varieties. BAP enhanced growth and development of axillary buds in *C. esculenta* var. *esculenta*, and this has been documented in a previous study (Chung and Goh, 1994). Seetohul et al. (2008) report the highest *in-vitro* multiplication rates of taro shoot tips in MS medium supplemented with 2 mg/L of BAP, which is agreeing with our findings. As observed in the current study, a higher concentration of BAP (6 mg/L) produced a fewer shoot compared to a lower concentration (El-Sayed et al., 2016). This could be attributed to the toxicity caused by high cytokinin concentrations, which causes a delay in shoot formation (Manju et al., 2017). During the multiplication stage, the requirement of cytokinin differs depending on the type of crop, explant, developmental phase, growth regulator concentration, and the interaction between growth regulators and the environment (Yokoya et al., 1999).

Both taro varieties used in this study successfully formed root when grown in MS media supplemented with different concentrations of IBA as well as in the absence of IBA. Roots are essential to plants as they supply water and nutrients to plants (Schiefelbein et al., 1997). An earlier study reports better rooting in taro shoots in half-strength MS medium supplemented with 1.5 or 2.0 mg/L of naphthaleneacetic acid (NAA) than in half-strength MS medium supplemented with 1.5 or 2.0 mg/L IBA at 15 days of culture (Behera and Sahoo, 2008). The present study showed that MS (4.4 g/L) premix supplemented with 0.5 mg/L of IBA was the best concentration for rooting in both taro varieties evaluated. The application of auxins to micro-propagated shoots could intensify the production of the root by increasing the endogenous contents of enzymes (Asghar et al., 2011). Another study reports auxins induced complication in lateral root formation through repetitive cell division (Liu et al., 2002). In the present study, the roots were of a shorter length in IBA supplemented medium than those induced in media with no IBA, possibly due to inhibition of shoot bud formation at elevated IBA concentration and arrest of root production as the auxin in the root primordial is shifted from the apex shoot (Ozel et al., 2006).

A previous study established that plants could be transplanted when they have rooted, but good results are achieved if the plants do not have too many long roots but have a greater number of roots (Singh et al., 2012). Despite initial difference in root length all the plants developed well during the acclimatization. The present study demonstrates that supplementation of MS media (4.4 g/L) with 2.0 mg/L BAP and 0.5 mg/L IBA enhance better shoot and root induction, respectively.

Conclusion

This study has developed an efficient regeneration protocol for Dasheen and Purple Wild, two common taro varieties in Kenya. The upper part of the corm and the base of the petioles were used as the explants. This protocol is a doorway for future studies on taro in tissue culture and facilitates rapid multiplication of disease-free genetically uniform taro varieties for commercial uses. The protocol can also be useful in the taro transformation process, a means for rapid multiplication of genetically engineered plants improved for different traits of agronomic importance.

ABBREVIATIONS

MS, Murashige and Skoog media; **BAP,** 6-benzylaminopurine; **IBA,** indole-3-butyric acid.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of light quality on *in vitro* germination, seedling growth and photosynthetic pigments production in wheat (*Triticum aestivum* L.)

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Due to its economic importance, wheat (*Triticum aestivum* L.) has been the subject of most breeding studies, most of which having as a starting point the *in vitro* cultures required for initiating different cell and tissues cultures such as protoplasts, ovules and pollen cells. All these procedures are used to obtain new varieties or hybrids regarding the improvement of different qualities such as higher productivity, more resistant towards pests and diseases, and to handle different environmental stress factors. This study presents specific results in attempt to optimize the efficient initiation and growth of wheat plantlets into *in vitro* cultures, by analysing the effects of different light wavelengths for the growing phase. The results showed that the vegetation stage of *in vitro* plantlets is highly influenced by the light wavelength, which may either stimulate a considered normal growth (in white artificial light) or contrary to decrease it and being relevant for maintaining wheat gene banks as *in vitro* culture for a long time (in red artificial light). The natural light may support the initiation of callus generation and protoplast cultures. The efficient use of light may further contribute to the cost efficiency of the process, ensuring the reduction of the carbon footprint of biotechnological protocols.

Key words: *In vitro*, *Triticum aestivum*, light, amelioration, seedlings, germination.

INTRODUCTION

Wheat is one of the main raw materials for the production of a wide range of food products, bioethanol, etc. Grains and products have long shelf life, and can be transported for a long time and distances without affecting their quality. This plant is one of the most important cereal crops and the most cultivated for the entire quantity of

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food required by humans and animals (Oyewole, 2016). Recently, Egyptian researchers have conducted studies to increase wheat productivity in order to minimize the gap between production and consumption by increasing the cultivated area (Rady et al., 2019). According to FAO data, the globally harvested wheat area has decreased by more than 7 million hectares (3.66%) less in the last 10 years, and wheat production during same period increased by more than 53 million tons (7.32%) (FAO, 2020). This increase is primarily due to the progressively productive varieties improved in specialized laboratories, and the reduction of the harvested area is a method to improve agricultural practices and decrease the carbon footprint. A growing interest in obtaining progressive productive varieties and hybrids can be noticed especially due to climate change effects on food security at the global level. This political interest at the global level is substantiated by the continuous growth of the world's population and on the other hand due to the adoption into agricultural practices of environmental protecting rules. Under such circumstances, each country as a Party to the Plant Treaty needs to address national surveys on all plant genetic resources for food and agriculture (PEGRFA) and, prioritizing their efforts in the quest to fight for food security and allow full access to genetic resources (Antofie, 2011). An example include the wheat case in Romania (Antofie and Sava, 2018).

Among the physical factors, the light studies on wheat morphogenesis was considered as extremely important. Thus, the efforts of researchers in the field of photomorphogenesis are aimed at the production of plants under artificial lighting, upon understanding the entire process of development. Under these circumstances, a complex knowledge approach to gain access to agricultural, biology and physiology features of the species development as well as considering the deep molecular mechanisms for responding to different environmental factors, has been applied to improve wheat genetic diversity and particularly to understand the growth development of wheat depending on lighting conditions. It has been proven that the blue light reduces cell expansion and consequently it inhibits leaf growth and stem elongation (Goto, 2003). Scientists have discovered the effects of red and blue light on the acquisition of the leaf shape, plant development and the accumulation of high levels of antioxidants such as phenolic compounds. Thus, red light stimulated the growth of the lettuce plant, and blue light was effective for the accumulation of phenolic compounds (American Society for Horticultural Science, 2013).

Thus, low intensity or lack of light of a certain wavelength negatively influences crops morphogenesis. Red and blue optical spectra with wavelengths between 640 and 660 nm and between 430 and 460 nm, were most effective for plants growth (Tertyshnaya and Levina, 2016). Light emitting diode known today as LED, accelerates growth and physiological processes in plants, in addition to reducing lighting costs. In the propagation phase, the red light stimulated the elongation of the shoots in *Vanilla planifolia* Andrews, as well as the synthesis of chlorophyll. During the rooting phase, the blue light stimulated rooting (that is, the number of developed roots) as well as leaf development (that is, the number of leaves). The treatments with different LEDs quality of light, increased the synthesis of photosynthetic pigments. The integration of these results into *in vitro* protocols may further contribute to the improvement of the micropropagation process of any species (Ramirez-Mosqueda et al., 2017).

Studies on the effect induced by different light intensities on germination and *in vitro* growth in wheat have been successfully conducted (Yao et al., 2017), but information on the effect of light with different wavelengths on this plant species *in vitro*, are very few. The high light intensity determined a high photosynthetic activity, increased biomass production and development efficiency. At lower light intensities and at blue and red light, separately, the results were antagonistic, offering an alternative for maintaining *in vitro* plants for longer period of time. A high level of blue light prolonged the juvenile phase. Both blue and red light altered the starch and protein contents into plantlets. Lights with different wavelengths are effective for experimental wheat cultivation and can be used to optimize growing conditions and manipulate metabolism, productivity and quality (Monostori et al., 2018).

Knowing the importance of *in vitro* cultures as a precursory phase to today plants breeding, as well as the fact that not all types of vegetative tissues are suitable for the initiation of cells and callus cultures, the purpose of our studies was to improve the efficiency of initiating and cultivating wheat into *in vitro* conditions. To this end, the analysis is on the effects induced by the light with different wavelengths, on the germination and growth of *in vitro* cultures of wheat (*Triticum aestivum* L). The objectives of the study was to find out which type of light stimulates germination, seedlings growth and development of wheat plantlets, to achieve as soon as possible, an *in vitro* plantlets collection, to be accessed for further biotechnological procedures (such as callusogenesis, protoplasts cultivation, and micropropagation). Using different lighting techniques proposed in this study, it is possible to shorten the time needed to maintain *in vitro* plantlets, to acquire performance and cost efficiency of the process in terms of energy consumption also. As a consequence, the study also tries to integrate into old biotechnological protocols, new approaches related to energy saving, lowering the carbon foot print and limiting environmental pollution.

MATERIALS AND METHODS

The plant material consisted of caryopses of *T. aestivum* L. varie

'CCB INGENIO C1', provided from commercial market. This variety was chosen after the analysis of germination capacity (GC) parameter (the percentage of seeds that would normally germinate under optimal conditions for the species). It was determined that the value of GC was 96% in 15 days, considering it is suitable for the use of the caryopse lot from which it came, for the initiation of *in vitro* culture.

Sterilization

The wheat caryopses was sterilized with 5% sodium hypochlorite solution of Domestos (commercial bleach).

Culture medium

The culture medium used was the basic Murashige-Skoog liquid (1962) (MS62) (Murashige and Skoog, 1962), modified, hormone free, pH =5.6.

Cultivation technique

Technique applied was "Blidar" type filter paper bridges (Blidar, 2004, 2014), which allows caryopses a better access to nutrients from liquid culture media for 21 days.

Growing conditions

The growth room provided low humidity (under 20%), a temperature of 26°C during the day and 25°C during the night with a photoperiodicity of 16 h at day and 8 h at night, under continuous ventilation.

Experimental procedure

In each culture, a single caryopsis on the 'Blidar' filter bridge was introduced to each vessel; after that, the culture vessels formed four experimental groups, dependent on the light conditions as follows: V_0 – White fluorescent light (λ = 400-700 nm); V_R – Red fluorescent light (λ = 610-700 nm); V_B – Blue fluorescent light (λ = 450-500 nm); V_N – Natural light. Each experimental lot consisted of 150 culture vessels.

Measurements, observations, and data analysis

During the whole experiment (of 21 days), measurements at 7, 14 and 21 days were performed, taking into account several morphological and physiological aspects. The following parameters were investigated during this experiment: root length (mm), number of roots, coleoptile length (mm), true leaf length (mm) and quantity of pigments at leaf level (µg pigment/g of sample sp): chlorophyll *a*, chlorophyll *b* and carotenoids (carotene, xanthophyll). The amount of pigments with photosynthesis role, accumulated at foliar level, was measured using a spectrophotometer (PG Instruments model T80+), according to the Moran (1982), the calculation formulas being those of Wellburn (1994).

Statistical analysis

V_0 was considered as the control group and the recorded values were taken as references for other experimental variants. All statistical analyses were made using Microsoft Excel; values are significantly different at $P<0.05$ according to the Student's t-test. The experiment was repeated three times, in the same conditions, using caryopsis from the same genotype.

RESULTS AND DISCUSSION

At day 7, after starting the experiment, the seed group enlightened with natural light (V_N) showed the highest values of all the following parameters: roots, coleoptiles and leaves being the best developed compared to all other experimental variants and compared to the control group (V_0) (Figure 1A and Table 1). On the contrary, the weakest results were recorded for the plants enlightened with blue light, except for the length of the leaves, the smallest growth increases were marked on red light, the difference being statistically significant. It is more proven that the germination of some seeds is influenced by light quality. The quality of the light influences the wheat growth rate even under continuous light at a constant temperature. In addition, the red light can play an important role in regulating the rate of wheat development, independent of the photoperiod (Kasajima et al., 2007). The blue light at the end of the visible light spectrum is active in promoting germination. Light-sensitive seeds will become ready for germination in any light or dark conditions, other conditions being appropriate, and the function of light seems to be only that of the final stimulus, either activating or inhibiting. The red region (= 650 nm) of the spectrum is most effective in stimulating the germination of *Lactuca* seeds, and the dark red radiation in the 730 nm region inhibits germination (Flint and Mcalister, 1935). The smallest increase was recorded for the leaf length parameter in the variant enlightened with red light (V_R) which showed an unfavourable difference of 76.82%, compared to V_0 (Table 1).

According to specialized studies, it is known that of the entire package of pigments, chlorophylls *a* are the most important pigments due to the fact that without them the photosynthetic process cannot take place (Sumanta et al., 2014). In the event of acclimation of *in vitro* plantlets, their presence in thier highest amount has a beneficial effect on the easier transition, from heterotrophic to autotrophic nutrition. For this reason, the amount of pigments with a role in photosynthesis has been studied and analysed.

According to the results on the first date of observations (7 days), of all the types of lights used, in absolute values, the blue light (V_B) most effectively stimulated the production of pigments; compared to control V_0, the chlorophyll *b* content was higher by 4.01% and the carotenoids by 22.69% (Figure 2A). This process can be explained by the fact that blue light, having a shorter wavelength and a higher frequency, was more easily absorbed by plants, thus intensifying the synthetic process of these pigments, even if the nutrition of *in vitro* plantlets was heterotrophic (Lesar et al., 2012). It should

Figure 1. General image of *in vitro* seedlings of *Triticum aestivum* enlightened under different lighting conditions, where: V_0 - white fluorescent light; V_R - red fluorescent light; V_B - blue fluorescent light and V_N - natural light, at 7 days (A) and 21 days (B) from the moment of initiating the experiments.

be noted that for all 3 variants, V_R, V_B, V_N, chlorophyll *a* recorded a lower value compared to the control group. The lowest values were highlighted in the version enlightened with natural light (V_N), where the pigments had lower increases than the control.

From a morphological point of view, artificial white light (V_0) and natural light (V_N) led to rapid growth of wheat seedlings, in some cases exceeding the available space of the used vials (Figure 1A). The group enlightened with natural light (V_N) due to the lack of differentiation may be optimal for the initiation of callus, cells, or protoplast cultures. Callus is an unorganized tissue or mass of cells, proliferated by undifferentiated cells (Suffness, 1995). Thus, young, and undifferentiated plants can be initiated from callus and/or cell cultures or protoplasts. At the end of 7 days, the highest reactivity was recorded for seedlings enlightened by the natural variant (V_N).

In the case of 14 days of observations, oscillations were noticed. The highest values being recorded both for the control variant (V_0) and the variant enlightened with fluorescent red light (V_R), depending on the analysed biometric parameter. Thus, the best overall results were recorded in the variant enlightened with white, fluorescent light (V_0) because it recorded the highest values of the roots and leave length (Table 1). The weakest results of the analysed parameters were highlighted in blue, fluorescent light, the difference being significant. As it is known, the coleoptiles of monocotyledons are sheath-shaped leaves whose role is to protect the embryonic bud. This leaf is pierced by the embryonic bud, and the moment it ceases to play its role, its growth is either inhibited or stopped, so that in a few days it enters senescence, and then into necrosis (Davet, 2004). The lowest increase was recorded for the length parameter of the leaf enlightened with blue light (V_B) with a very significant statistical value of -69.96%, compared to V_0 (Table 1). For wheat, the optimal conditions can be defined as hydration in the dark, conditions that wheat seeds experience in the field during sowing. The degree of inhibition of germination by blue light was dependent on light intensity (Jacobsen et al., 2013).

At day 14, there is an increase in the amount of analysed pigments, blue light (V_B) being the most effective, the accumulation increases compared to the control being 109.02% for chlorophyll *a*, 107.84% for chlorophyll *b* and 136.8% for carotenoids (Figure 2B).

Table 1. Statistical data on the average number of roots (A), root length (B), coleoptile length (C) and leaf length (D) in *Triticum aestivum* seedlings enlightened with V_0 - white fluorescent light; V_R - red fluorescent light; V_B - blue fluorescent light and V_N - natural light, during the 21 days of vitro culture.

Parameter	Days	Control group	Statistic data	Experimental variants	Statistic data		Statistical significance
			X ± Sx		X ± Sx	p (testul *t*)	
				V_R	4.083 ± 1.78	0.044	**
	7		5.5 ± 1.45	V_B	4 ± 1.41	0.020	**
				V_N	6.73 ± 1.19	0.037	**
				V_R	5.3 ± 1.42	0.438	Ns
A. Roots no./plant	14	V_0	4.8 ± 1.398	V_B	2.875 ± 0.99	0.0036	***
				V_N	6.1 ± 1.29	0.044	**
				V_R	4.5 ± 0.76	0.777	Ns
	21		4.6 ± 0.699	V_B	3.5 ± 0.93	0.0157	**
				V_N	5.8 ± 0.92	0.0044	***
				V_R	13.25 ± 4,14	0.00209	***
	7		20.083 ± 5.32	V_B	8.091 ± 4.61	0.00001	***
				V_N	25.73 ± 6.13	0.029	**
				V_R	21.4 ± 4.74	0.330	Ns
B. Roots length (mm)	14	V_0	23.7 ± 5.498	V_B	10.375 ± 5.18	0.00008	***
				V_N	19.1 ± 3.21	0.0379	**
				V_R	16.25 ± 5.365	0.188	Ns
	21		20.3 ± 7.13	V_B	9.75 ± 5.599	0.0029	***
				V_N	20 ± 4.37	0.911	Ns
				V_R	10.17 ± 3.93	0.00014	***
	7		17.58 ± 3.99	V_B	9.36 ± 3.38	0.00003	***
				V_N	38 ± 5.69	0	***
				V_R	14.4 ± 2.72	0.0058	***
C. Coleoptile length (mm)	14	V_0	18.4 ± 2.99	V_B	8.875 ± 2.47	0	***
				V_N	33.2 ± 7.81	0.00013	***
				V_R	12.375 ± 2.92	0.0029	***
	21		18.7 ± 4.62	V_B	14.75 ± 7.34	0.211	Ns
				V_N	30 ± 3.89	0.00002	***
				V_R	12.42 ± 8.31	0.0001	***
	7		53.58 ± 24.93	V_B	14.18 ± 8.4	0.00016	***
				V_N	72.36 ± 15.77	0.042	**
				V_R	47.3 ± 24.47	0.0919	*
D. Leaf length (mm)	14	V_0	69.5 ± 30.78	V_B	20.87 ± 11.06	0.0006	***
				V_N	58.4 ± 35.32	0.464	Ns
				V_R	43.25 ± 16.11	0.829	Ns
	21		45.3 ± 23.45	V_B	21.75 ± 12.98	0.0168	**
				V_N	71 ± 22.326	0.022	**

X ± Sx [mean ± standard deviation]; depending on the value of *p* (significance of the difference compared to the control group): ns – not-significant difference (p> 0.1); *Weakly significant difference (0.05≤p <0.1); **Significant difference (0.001≤p <0.05); ***Very significant difference (p <0.01).

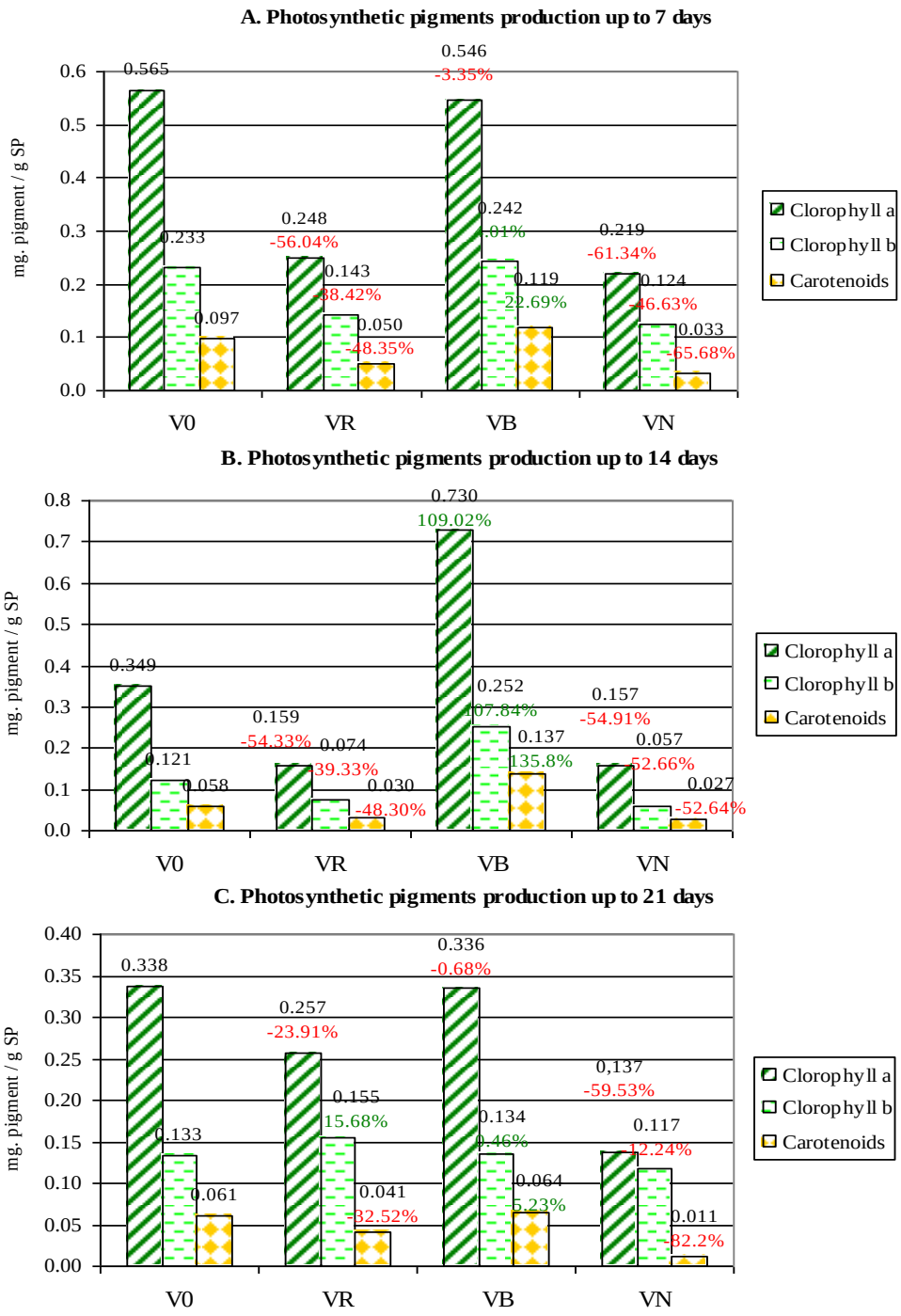

Figure 2. A general comparison of the amounts of chlorophyll pigments in the leaves of *Triticum aestivum* seedlings at 7 (A), 14 (B) and 21 days (C) from the initiation of *in vitro* cultures.

The variants enlightened with natural light (V_N) and red light (V_R) showed negative increases similar to the control variant (V_0), both on the first date of the observations and on the second. In the latter case, the differences compared to the values of the V_B group being highly significant.

On this experimental date, the best overall results were recorded in the variant enlightened with white artificial light (V_0) because it recorded the highest values of root and leaf length, which is effective for possible acclimation procedures. It should be noted that among the experimental variants enlightened with coloured light, red

light induced the largest increase in the range of 7 to 14 days, but in absolute terms, the recorded data remained lower than natural light. Blue light did not play such an important role in growing but is useful for storing *in vitro* plantlets for a long time in the containers.

On the 21st days of *in vitro* culture, the highest values for parameters number of roots, coleoptile length and leaf length were recorded in the variant enlightened with natural light (V_N), where the highest increase was 60.43% for the length of the coleoptile, statistically significant difference compared to the control group (Table 1). Similar to the results obtained at 14 days, the lowest values for almost all parameters, namely, number of roots, root length and leaf length were for the variant enlightened with blue light (V_B) (Figure 1 B). The lowest increase was -59.99% for leaf length.

The concept of using sunlight for the micropropagation systems is suggested as a way to reduce tissue culture costs. Significantly, more shoots were produced by the *Musa acuminata* seedlings grown in a sunlit room during summer, with photosynthetic photon flux densities (PPFD) ranging up to 570 µmol m^{-2} s^{-1}, at temperatures of 23 to 30°C and photoperiods of 12 to 16 h, than seedlings under artificial light in a growing room which ensures controlled conditions of a constant PPFD of 65 µmol $m^{-2}.s^{-1}$, at temperatures between 23 and 29°C and 16 h photoperiod (Kodym and Zapata-Arias, 1999).

It is well known that the energy of photons is inversely proportional to the wavelength of light radiation, so that the photons of blue radiation have a higher energy compared to those of red radiation and determine the achievement of a photosynthetic maximum. Red radiation is the complementary colour for chlorophyll green pigments, as the amounts of red radiation that have the lowest mass are better absorbed by the chlorophyll molecules of most plant species (Inada, 1976). By knowing these physiological characteristics, it was expected that the blue light (V_B) would lead to the accumulation of a lower amount of chlorophyll *a*, a phenomenon was found at day 7. A similar situation was identified for the amount of chlorophyll *b* in the control variant and in the blue light, the amount of pigment being approximately 0.134 g. The amount of carotenoids in variant V_B showed a positive increase of 5.23% compared to V_0 (Figure 2C). For the 3 data of observations, at 7, 14, 21 days, natural light (V_N) led to negative accumulation increase in all pigments compared to the values recorded in the control group, on the last experimental date, by 59.53% for chlorophyll *a*, 12.24% for chlorophyll *b* and 82.2% for carotenoids. Due to the fact that overall, the highest quantities of assimilating pigments (chlorophylls and carotenoids) accumulated in the leaves were recorded in seedlings exposed to blue artificial light, it is recommended to use it in the processes of acclimation to the septic environment, as these seedlings may have the greatest adaptability to the change of nutrition from the heterotrophic to the autotrophic type during the acclimation process.

On the last date of the observations, the biometric results showed that the seedlings enlightened with natural light (V_N) had the most numerous roots, the highest number of leaves and coleoptiles. In case it is desired to initiate wheat root crops, along with phytohormones stimulating rhizogenesis, a lighting can be used to increase the stimulation of root proliferation and growth, natural light. The variant enlightened with white artificial light stimulated each experimental date, both the growth and development of the seedlings. Red artificial light (V_R) and blue artificial light (V_B) can be used to maintain these *in vitro* seedlings for a long time *in vitro*. Red light has also proven effective on protoplasts from wheat leaves (*T. aestivum*), as they respond to short irradiation of red light by increasing their number (Fallon et al., 1993).

Similar results on leaf growth, chlorophyll content and root branching were obtained when using LED light. In *Cymbidium*, red light promoted leaf growth, but decreased chlorophyll content. It was reversed by the blue light; root branching was comparable under red plus blue LEDs and in fluorescent lighting systems (Tanaka et al., 1997). The morphometric analysis of the palisade cell micrographs in *in vitro* cultures of *Betula pendula* showed that the area of the functional chloroplast was the largest in the chloroplasts of leaves exposed to blue light and the smallest in those exposed to red light (Saebø et al., 1995), which confirms that vitroplants grown in blue light are more easily adaptable to acclimation.

Conclusion

Overall, the most effective variant on the proliferation and root growth of the coleoptile and leaf in wheat seedlings, also on the quantities of pigments accumulated into the leaves, was represented by the use of white artificial light. For wheat seedlings germinated under these conditions, the high degree of growth and branching recommends the use of white light, mainly for *in vitro* subcultures or for micropropagation, and secondarily in the processes of acclimation to the living septic environment and hydric stress; the seedlings being kept for a short period of time *in vitro*, and the optimal time for mending or acclimation was 14 days.

When the red light was used, two advantages were noted, namely: (1) the possibility of keeping wheat into *in vitro* culture for a longer period of time, as the seedlings did not show any sign of senescence and/or necrosis and (2) economic advantage, because this technique does not require the use of growth inhibitory hormones (that is, abscisic acid) to slow the growth rate of *in vitro* plantlets, while maintaining vitality and thus the proliferative and growth properties.

When the blue light was used, wheat seedlings were obtained with the highest degree of accumulation of

photosynthesizing pigments, which favours the transition from *in vitro* to natural conditions (*in situ*), respectively for the transition from heterotrophic to autotrophic nutrition. From this point of view, it is recommended to use blue light, but not after the 14th day of vitro culture, when the maximum accumulation of pigments with a role in photosynthesis is reached.

Maintaining wheat *in vitro* culture for up to 14 days under natural light has proven to be successful in terms of growing in the direction of obtaining a source of quality germplasm to establish cultures of callus, cells in suspension or protoplasts. Also, natural light most strongly stimulates root and cauline proliferation and growth, but newly developed organs have a lower degree of differentiation than other working variants. The benefits of using this type of light are of economic nature as crops do not require artificial lighting, with a significant reduction in energy consumption.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Control of ginseng leaf black spot disease by endophytic fungi

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Ginseng plants associated with a number of fungi, several of which are reported to protect it from pathogens, thus improving plant growth. This study aimed to screen *Panax ginseng* leaves for endophytic fungi and to assess these fungi for their efficacy to inhibit ginseng black spot disease caused by *Alternaria panax*. A total of 256 endophytic fungal isolates were obtained from *P. ginseng* leaves. Most of the fungal isolates belonged to *Chaetomium*, *Nemania*, *Xylaria*, *Nodulisporium* and *Alternaria*; the others were not identified. One isolate *Chaetomium globosum* (FS-01), inhibited on *A. panax* causes black spot disease of ginseng, suggesting that FS-01 can be a potential biocontrol resource for control of ginseng black spot diseases. This is the first report on *C. globosum*, an endophytic fungus as biocontrol agent for ginseng black spot.

Key words: *Panax ginseng*, inhibitory effect, *Chaetomium globosum*, biological control.

INTRODUCTION

Endophytic fungi are defined as fungi that spend all or part of their life cycle within plant tissues or organs. Some fungal endophytes do not cause any harm to their host plants, and they are commensals or mutualists (Hyde and Soytong, 2008; Rodriguez et al., 2009). The mutualist fungal endophytes play an important role in improving plant growth and protecting plants against pathogens (Backman and Sikora, 2008; Kumar and Kaushik, 2013). Endophytic fungi as a biological control have received much attention in the past 20 years (Bacon et al., 2001; Porras-Alfaro and Bayman, 2011).

Black spot disease caused by *Alternaria panax* is one of the most severe diseases of ginseng; it seriously affects the yield and quality of this crop (Sun et al., 2017). Currently, chemical fungicides are used to control this disease. However, spraying leave pesticide residues in the ginseng plants. Biological control using endophytic fungi has been investigated in recent years as an alternative to pesticide control (Park et al., 2017).

About 25% of crops worldwide are infected by plant pathogens each year according to the FAO (Schmale and Munkvold, 2009). Chemical pesticides are widely used to control plant diseases caused by fungi. Biological control for plant diseases is an alternative. Mutualist endophytic fungi live in plants, but do not cause harm to the host. Some control diseases by improving the resistance of the plant (Schulz et al., 2002; Silva et al., 2006; Strobel, 2006). Some endophytic fungi can secrete biologically

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Table 1. Antagonistic activity of *Chaetomium globosum* against five strains of *Alternaria panax*.

No.	Inhibition zone (mm)
FS-01	11±0.031
FS-02	7±0.056
FS-03	9±0.024
FS-04	5±0.037
FS-05	6±0.029

active substances, which enhance the host plants' resistance to pathogenic infections (Katoch and Pull, 2017).

In this study, fungi isolated from the leaves of healthy ginseng were screened for their antagonistic ability toward *A. panax,* the causative agent of ginseng black spot disease.

MATERIALS AND METHODS

Isolation

Asymptomatic leaves were collected from 15-year-old-healthy ginseng from a forest in Fusong county, Jilin province, latitude 41°42′N and longitude 127°01′E. The leaves were surface-sterilized according to the Kusari's protocol (Kusari et al., 2013). In brief, leaves were washed using distilled water, followed by treatment with bleach for 3 min which is buffer and 0.1% mercury for 1 min and then rinsed in sterile distilled water three times. The water from the final wash was collected and plated onto PDA to confirm that the surface sterilization was sufficient to remove surface contaminants. The surface sterilized leaves were cut into 4 to 5 pieces (5×5 mm^2) and plated on potato dextrose agar (PDA) containing 100 µl/ml streptomycin. The samples were incubated at 25°C in an incubator. During hyphal growth, hyphal tips were picked with a sterilized inoculation needle and transferred onto PDA plates. Subcultures were maintained at 4°C (Tejesvi et al., 2011).

Ginseng pathogens

An *A. panax* Whetzel (1912) culture was obtained from the Plant Pathology Laboratory of Jilin Agricultural University (Accession No. JL910032). Subcultures were maintained on PDA plates at 25°C.

Pathogenicity test experiments were conducted to ensure that the endophytic fungi were nonpathogenic on ginseng (Arnold, 2007).

Screening experiment *in vitro*

The inhibitory effects of the isolated fungal strains on *A. panax* were assessed through a procedure using the dual_culture method (Dasari et al., 2011). The interaction of the strains with the mycelial growth of *A. panax* was observed under the microscope.

Morphological identification

The morphological characteristics of Strain FS-01 were observed after incubation on PDA at 25°C for seven days, and identified using light microscopy according to the morphological characteristics of its colony and spores.

18S rDNA sequence analysis

The 18S rDNA sequence was amplified using polymerase chain reaction (PCR), which were amplified using the fungal universal primers ITS1 (5'-TCCGTAGGTG-AACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Amplified PCR products were cloned and sequenced according to the Pan's protocol (Pan, 2013), and nucleic acid identified was determined using NCBI BLAST (http://www.ncbi.nlm.nih.gov).

Greenhouse experiment

This experiment was conducted in the greenhouse (16 h of sunlight at 14 to 28°C, relative humidity 70-80%) with flower pots [30 cm (diameter) × 20 cm (height)] filled with 3000 g of soil matrix. *Chaetomium globosum* was applied at 1×10^6 mL^{-1} spore suspension to the five leaves per plant, a total of 10 ginseng plants, with three replications. The leaves of ginseng were inoculated with suspensions of the FS-01 strain with addition of 0.02% Tween 80 using a watering can, 1 day before the *A. panax*. Subsequently, suspensions of the *A. panax* and the FS-01 strain were prepared with sterile distilled water using sterile loops. The conidial concentration used for *A. panax* was 1×10^5 conidia/ml, whereas for the FS-01 strain a 1×10^6 conidia/ml was prepared. Control plants were inoculated with the *A. panax* using a watering can, negative control plants were inoculated with sterile distilled water. All plants were incubated under temperature maintained at 25±2°C in a greenhouse for 25 days. Disease index and control efficiency were calculated with the following formulas:

Disease index (%) = ∑(number of infected plants × group) / (total plant number × highest group) × 100

Control efficacy (%) = Disease index of control - Disease index of treated group/disease index of control × 100.

RESULTS

Screening of antagonistic strains against ginseng black rot pathogens

A total of 256 fungal cultures were isolated through two rounds of leaf sample screening, according to the morphological characteristics of the colonies. Five of these showed prominent antagonistic activities against *A. panax in vitro* (Table 1). Strain FS-01 had an 11 cm of inhibition zone against *A. panax* in the dual-culture test (Table 1), which was the largest inhibitory activity among the isolates. FS-01 caused morphological changes to the mycelia of *A. panax* that could be characterized as being degraded (Figure 1A) or twined (Figure 1B) under microscope.

Morphological identification of endophytic fungi

The colonies of isolate FS-01 were compact, gray on

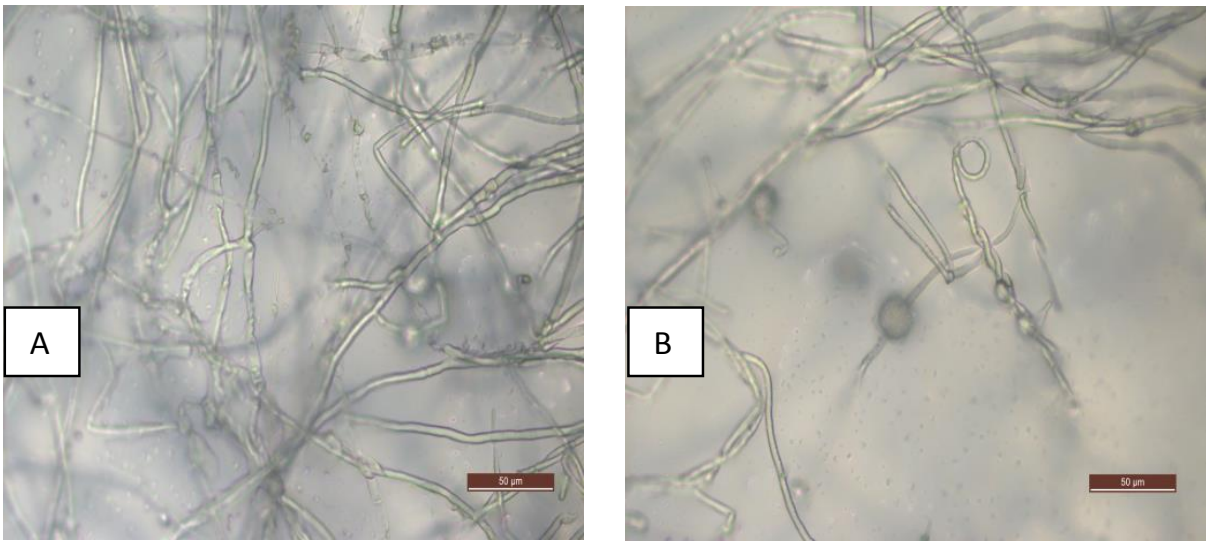

Figure 1. Inhibitory effect of Strain FS-01 on *Alternaria panax* using dual-culture. A: hyphae of *Alternaria panax* dissolved; B: hyphae of *Alternaria panax* twined.

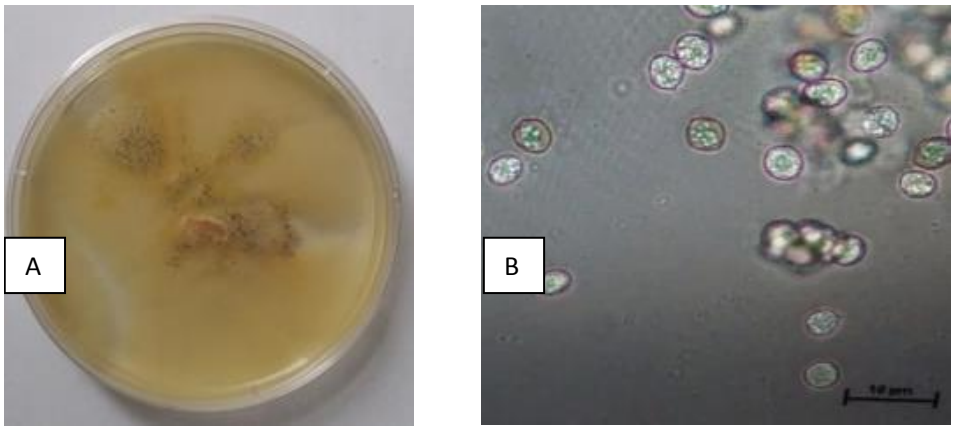

Figure 2. The characteristics of the colony and conidia of FS-01 strain on PDA.

PDA medium but later became yellowish green in the following days (Figure 2A). Isolate FS-01 was identified as a member of the Chaetomiaceae family according to its morphological characteristics of the colonies. The ascocarp of isolate FS-01 was spherical or oval, ascus stick or handle shape, ascospores brown, lemon, thick-walled, bulging at both ends, with one tremata on the top (Figure 2B).

Identification of 18S rDNA sequence

PCR products of 578-bp were obtained from amplification of the 18S rDNA of the genomic DNA of strain FS-01. Sequence analysis showed that strain FS-01 shared 99% identity with a number of *C. globosum* in the NCBI database (Accession No. KX421415.1). Phylogenetic dendrogram was constructed by using 18S rDNA sequences, and it clearly showed that strain FS-01 clustered with members of the genus *Chaetomium* (Figure 3). Strain FS-01 was identified as *C. globosum* based on the results of the 18S rDNA sequence analysis and the morphological characterization.

Greenhouse experiment

The results of the greenhouse inoculation experiment showed that disease index of plants inoculated with strain FS-01 was 41.9 lower than that of control, indicating that

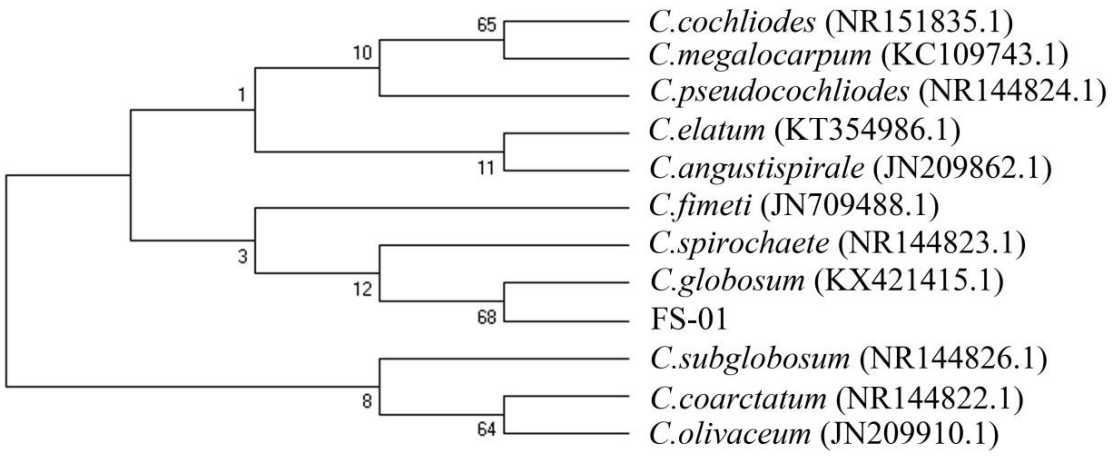

Figure 3. Phylogenetic dendrogram of strain FS-01 based on 18S rDNA sequence.

Table 2. Effect of *Chaetomium globosum* Strain FS-01 on black leaf spot disease of ginseng as assessed 25 days after inoculation.

Treatment	Parameters	
	Disease index	Control efficacy (%)
Control plants	82.2±0.079	0
Inoculated plants with strain FS-01	41.9±0.035	49

it had control over ginseng black spot disease (Table 2).

DISCUSSION

Endophytic fungi living in host plants are valuable natural resources that can be exploited as biocontrol agents because of their beneficial effects on host plant growth (Li et al., 2012). They can induce phytochemical production and defense resistance against pathogens (Zhang et al., 2014). Therefore, the biocontrol agents of *C. globosum* isolated from gingseng leaves were investigated.

C. globosum is a fungus that belongs to the Ascomycota, Pyrenomyetes Phylum, Sordariales Order, Chaetomiaceae Family, *Chaetomium* Genus (Kirk et al., 2008). In recent years, some *Chaetomium* were shown to strongly inhibit black pepper mould, gray mold of strawberry, wilt of tomato, basal rot of corn, and tan spot in wheat (Soytong et al., 2001; Istifadah and McGee, 2006). *C. globosum* was the most reported biocontrol fungus, which has strong inhibitory effects toward *Venturia inaequalis*, *Fusarium oxysporum,* and *Setosphaeria turcica* (Cullen and Andrews, 1984; Walther and Gindrat, 1988). *C. globosum* have been exploited as biocontrol agents with various mechanisms including mycoparasitism, antibiosis, induced resistance, and competition for nutrients (Park et al., 1988). However, here we present the first report of *C. globosum* as a biocontrol agent for ginseng black spot.

This study was conducted to screen biocontrol efficacy of the endophytic fungi from healthy leaves of ginseng for the control of ginseng black spot disease. The FS-01 strain had obvious inhibition on the incidence and reduction of severity of ginseng black spot. It was speculated from these results that FS-01 strain reduced diseases by producing some antifungal substances, but this will require additional work to demonstrate. Therefore, FS-01 strain has potentials to serve as a biocontrol agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of intercropping aerobic rice with leafy vegetables on crop growth, yield and its economic efficiency

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Aerobic rice production system provides a sustainable alternative to the traditional rice cultivation. To evaluate the agronomic and economic effect of intercropping aerobic rice with four leafy vegetables, field experiments were conducted at University of Agriculture Science Bangalore research station, India during 2017 and 2018. The experiments consisted of 9 treatments with 4 replications and, a Randomized Completely Block Design was applied. The treatments were as follows: Intercropping (IC_1): rice+amaranth; IC_2: rice + coriander; IC_3: rice + spinach, and IC_4: rice + fenugreek plus other 5 treatments of solecrops (SC), SC_5: rice, SC_6: amaranth, SC_7: coriander, SC_8: spinach, and SC_9: fenugreek. Results showed that intercropping produced significantly better plant growth and higher yields than sole crops. The rice-spinach intercrop produced highest rice grain yield (7,651 kg ha^{-1}), vegetable yield (25,508 kg ha^{-1}), land equivalent ratio (2.13), rice equivalent yield (16,153 kg ha^{-1}), production efficiency (107.69 kg day^{-1}), area time equivalent ratio (1.23) and system harvest index (0.77). Net return and benefit cost ratio of rice-spinach intercropping were also higher than that of sole crops. This suggests that intercropping of aerobic rice with leafy vegetables can be productive and economically efficient.

Key words: Aerobic rice, intercropping, leafy vegetables, productivity.

INTRODUCTION

Rice is the second most widely cultivated cereal after corn and is staple food for more than half of world's population (CGIAR, 2016). Asia-Pacific region produces and consumes over 90% of world rice (Nirmala, 2017). In many parts of the world, rice is predominantly transplanted and flood-irrigated with standing water throughout the season. In Asia especially China and India, 75% of harvested rice is irrigated and lowland type. The

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flooded rice production system is usually preferred as a weed management strategy (Shaibu et al., 2015). Due to present day water resource crisis and high demand for irrigation of other crops, aerobic rice production has been introduced and adopted in several countries including India (Kadiyala et al., 2012; Priyanka et al., 2012).

Aerobic rice is a lowland rice planting system that involves growing drought tolerant high yielding rice varieties in non-flooded soils and with no puddling (Bouman and Toung, 2001; Patel et al., 2010). The aerobic rice has low water requirement as compared to lowland rice and can save about 45% water (Lampayan et al., 2010). Besides being water saver, aerobic rice production is often affected by several abiotic and biotic stress factors such as nutrient deficiencies (Jiban et al., 2019), nematodes (Kreye et al., 2009) and high weed pressure (Anwar et al., 2010; Kumalasari and Bergmeier, 2014). Consequently, these conditions lower yield potential of aerobic rice leading to heavy losses.

Intercropping is a practice that involves growing of two or more crop species at the same time in a field, and is traditionally used as an important strategy in sustainable agriculture (Bybee-Finley and Ryan, 2018). Intercropping is known to increase crop yields, reduce pests and disease and suppress weeds. To explore the potential of intercropping, aerobic rice has also been intercropped with other crops. For instance in Nigeria, farmers intercropped upland rice with cassava and vegetables (Okonji et al., 2012). Jadeyegowda et al. (2019) evaluated different aerobic rice intercropping systems and their effect on rice growth and yield. Intercropping of aerobic rice with watermelon alleviate *Fusarium* wilt by restraining spore formation and improving soil heath (Ren et al., 2008). Increased crop biomass helped in suppressing weed in aerobic rice when intercropped with vegetables (Habimana et al., 2019). The objectives of this study were to assess the effect of intercropping aerobic rice with leafy vegetables on growth, yield and to assess its economic efficiency.

MATERIALS AND METHODS

This study was conducted during two consecutive summer seasons in 2017 and 2018 at research experimental station of University of Agricultural Sciences, Bangalore (UASB), India. The experiment was laid out following RCBD design with nine treatments and four replications. The treatments were: Intercropping (IC_1): rice+amaranth, IC_2: rice + coriander, IC_3: rice+spinach, and IC_4: rice + fenugreek. The remaining 5 treatments were sole crops (SC), namely: SC_5: rice, SC_6: amaranth, SC_7: coriander, SC_8: spinach, and SC_9: fenugreek. Farmyard manure was applied to all plots at a rate of 10 tonnes ha^{-1} 15 days before sowing. The rice and vegetable seeds were directly sown into the soil and common fertilizers such as urea, single superphosphate and muriate of potash were applied. The experimental site had red sandy loam soil with pH 6.7, organic carbon 0.58%, available N 362 kg ha^{-1}, available P 43 kg ha^{-1} and available K 289 kg ha^{-1}. Anaerobic rice genotype MAS946-1 was used in this study. All treatments were managed until maturity and data were collected using five plant samples.

To compare performance of sole rice treatments with the other leafy vegetable intercrops, data on growth, yield and yield attributes were recorded and pooled for 2017 and 2018 and averages were examined through Least Significant Difference (LSD) test at 5% degree of significance. Leafy vegetable, rice grain and straw yield were expressed in ha^{-1} before the analysis of variance (ANOVA). The productivity of intercropping was examined by calculating several parameters. The Land Equivalent Ratio is used to decide which crop is suitable. It denotes relative land area under sole crop required to produce the same yield as obtained under a mixed or an intercropping system at the same level of management. It is the ratio of land required by pure crop to produce the same yield as intercrop. Land Equivalent Ratio (LER) was calculated following Willey (1979). Rice equivalent yield (REY) refers to the yields of different intercrops/crops which are converted into equivalent yield of any one crop based on price of the produce. This was calculated by considering the grain yield of component crops and the existing market price of aerobic rice crop and leafy vegetables components as following Verma and Modgal (1983). Based on REY and duration of the cropping system, production efficiency (PE) was also calculated and expressed as kg day^{-1} according to Habimana et al., (2019). It was based on the rice equivalent yield and duration of cropping system. Area time equivalent ratio (ATER) provides more realistic comparison of the yield of intercropping over monocropping in terms of time taken by component crops in the intercrop. It was used to compare yield advantages of intercropping components over a stand-alone cropping system, and was calculated following Hiebsch and Macollam (1980). The data on the system harvest index (SHI) in the intercropping experiment of rice-leafy vegetables was calculated as following:

$$SHI = \frac{\text{The economic yield of main crop + Economic yield of intercrop per unit area}}{\text{The biological yield of main crop + Biological yield of intercrop per unit area}}$$

To evaluate economic performance of different intercropping systems, gross and net returns were estimated as of Sujan et al., (2017a, b) in their studies. Benefit cost ratio (BCR) of components crop yield was calculated following the calculation of Bala et al. (2020), Sujan et al. (2021), and Sahota and Malhi (2012).

RESULTS AND DISCUSSION

Growth, yield and yield attributes of rice under intercropping system

Results for rice growth parameters, rice yield attributes, grain yield and straw yield have shown significant difference among the treatments (Table 1). Plant growth, rice grain yield (7,651 kg ha^{-1}) and straw yield (9,687 kg ha^{-1}) were highest in the IC_3 (rice + spinach), whereas rice sole crop showed lowest plant growth, yield, yield attributes and straw yield as compared to the intercrops (Table 1). The higher amount of rice grain yield in IC_3 could be as a result of better yield attributing characters such as number of productive rice tillers per hill (34.10), total number of grain per panicle (160.54) and thousand grain weight (23.72 g). Good performance of the IC_3 could

Table 1. Growth, yield, yield attributes of rice-leafy vegetables intercropping systems (Pooled data of 2017 and 2018).

Treatment	Plant height (cm)	No. of tillers hill^{-1}	TDM (g plant^{-1})	No. of productive tillers hill^{-1}	Total No. of grain panicle^{-1}	1000 grain wt. (g)	Grain yield (kg ha^{-1})	Straw yield (kg ha^{-1})	Vegetable yield (kg ha^{-1})
IC_1	67.71	35.3	136.96	31.17	144.14	21.11	6242	8275	14029
IC_2	61.42	31.91	116.23	25.99	125.27	18.39	5731	7936	11642
IC_3	71.22	38.49	150.4	34.1	160.54	23.72	7651	9687	25508
IC_4	64.11	33.11	125.29	29.9	137.48	19.74	6044	8103	13095
SC_5	57.29	28.88	110.55	25.6	119.64	16.94	5691	7278	-
SC_6	-	-	-	-	-	-	-	-	18708
SC_7	-	-	-	-	-	-	-	-	14784
SC_8	-	-	-	-	-	-	-	-	32405
SC_9	-	-	-	-	-	-	-	-	17318
S.Em.±	0.335	0.8	7.35	0.28	2.1	0.3	130.36	259.68	1426
CD (P=0.05)	1.043	2.4	22.9	0.9	6.56	0.95	406.15	809.03	4189

TDM: Total dry matter; S.Em: standard error of mean; CD: critical difference.

be attributed to better growth performance (Table 1). Besides, the large canopy of spinach produced minimum weed population in those plots, which increased the equilibrium thus benefiting the crop in maximum utilization of the accessible resources such as increased soil moisture availability during intercrop period. These results are in conformity with the findings of Mian et al., (2010).

Yield of leafy vegetables

Under intercropping, the yield of amaranth, coriander, spinach and fenugreek were 14029, 11642, 25508 and 13095 kg ha^{-1}, respectively (Table 1). However, when planted as a sole crop, leafy vegetables produced higher yield than when intercropped. The reduction in yield could be as a result of competition for resources during intercropping hence indicating that rice crop was dominant over the leafy vegetables (Oroka and Omororegie, 2007).

Rice equivalent yield (REY) and other efficiencies

All intercropping efficiencies, land equivalent ratio (LER), rice equivalent yield (REY), production efficiency (PE), area time equivalent ratio (ATER) and system harvest index (SHI) were significantly different among the treatments (Table 2). The LERs for all types of intercrops were higher than sole crop, thus indicating that intercropping rice crop with leaf vegetables was more beneficial than sole rice production. This is indicated by better growth and grain yield advantage (80-113%), which is exhibited under intercropping as indicated in Table 1. Higher LER could be due to better use of natural resources as previously indicated by Jabbar et al. (2009) and Udhaya and Kuzhanthaivel (2015). The REYs were higher in all the intercrops with rice-spinach intercrop producing the highest REY (16,153 kg ha^{-1}) (Table 2). High REY indicate the increased productivity in intercrops as compared to sole crop. These results confer with previous studies of Nagwa et al. (2014) and Rayhan et al. (2014). The maximum PE (107.69 kg day^{-1}) was found in rice-spinach intercrop. The findings showed that the intercrops components stayed in the field for a short time and leaf yields were also high resulting to high biomass production per day. Ibni et al. (2005) and Nazrul and Shaheb (2014) reported similar findings. The ATER values showed an average up to 23% in intercropping combination comparison with the sole rice cropping pattern. Intercropping rice with spinach also produced highest ATER of 1.23. Similar trends were reported in research studies of Mian et al., (2011) and Nagwa et al., (2014). The SHI, all intercrops showed higher values and HI of 0.42 for the sole rice crop. These results confer with the findings of Hugar and Palled (2008), Jabbar et al., (2009) and Mohan (2012).

Economic efficiency

Highest gross return (2,56,842 Indian Rupees (INR) ha^{-1}), net returns (INR 212,860 ha^{-1}), and benefit cost ratio (BCR = 5.84) were obtained when rice was intercropped with spinach (Table 3). Both sole rice crop and spinach produced lower gross return, net return and BCR as compared to rice-spinach combination. BCR increased in IC_3 mainly due to the increase in rice grain and straw yield under intercropping system. High aerobic rice yield

Table 2. Yield of companion crops, REY and other efficiencies under rice-leafy vegetables intercropping systems (Pooled data of 2017 and 2018).

Treatment	REY (kg day^{-1})	LER	ATER	SHI	PE (kg day^{-1})
IC_1	10919	1.85	1.04	0.71	72.79
IC_2	9611	1.8	0.97	0.69	64.08
IC_3	16153	2.13	1.23	0.77	107.69
IC_4	10409	1.83	1.01	0.7	69.39
SC_5	-	-	-	-	-
SC_6	-	-	-	-	-
SC_7	-	-	-	-	-
SC_8	-	-	-	-	-
SC_9	-	-	-	-	-
S.Em.±	702	0.08	0.021	0.009	4.12
CD (P=0.05)	2278	0.24	0.066	0.029	12.85

Rice equivalent yield (REY), land equivalent ratio (LER), area time equivalent ratio (ATER), system harvest index (SHI), production efficiency (PE).

Table 3. Economic efficiency of rice-leafy vegetables intercropping systems (Pooled data of 2017 and 2018).

Treatment	Gross returns (₹ha^{-1})	Net returns (₹ha^{-1})	Benefit cost ratio
IC_1	172228	131971	4.28
IC_2	156798	114541	3.71
IC_3	256842	212860	5.84
IC_4	169725	127688	4.04
SC_5	96348	56591	2.42
SC_6	93548	80558	7.2
SC_7	74271	59281	4.95
SC_8	162024	145309	9.69
SC_9	92214	77444	6.24

and leaf yield of vegetables, which in turn increased gross and net returns. Generally, intercropping was economically efficient as compared to sole crops. Similar results were also reported in pea-maize intercropping systems (Yang et al., 2018).

Conclusion

The results of this study showed that all four intercropping combination treatments were appropriate in relation to the stand-alone aerobic rice crop and leafy vegetable treatments. However, aerobic rice intercropping with spinach leafy vegetable has exhibited high production and economic efficiency with respect to biological yield, intercropping efficiencies and benefit cost ratio. Hence, intercropping could be recommended to aerobic rice growers in the studied area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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