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The Accumulation of Phenolic Compounds in Genetically Selected *Amaranthus hybridus* is Influenced by Endophytic Natural Growth Regulator

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Abstract: Amaranth (*Amaranthus spp*. L.) (*Amaranthaceae*), an endemic plant in Central and South America, grows worldwide, being cultivated in many temperate and tropical countries. Although several species of amaranth are frequently considered weeds, the plant is recognized as a food, constructive medicine, a source of protein and minerals livestock feed. The plant is widely cultivated, promoted, and increasingly consumed as a leafy vegetable and traditional medicine in Africa. Despite progressive genetic improvement and modern plant growing technologies, unfavorable climatic and ecological factors reduce the yield, and quality of the active plant botanicals. The role of bio-transformed endophytic microbial plant growth regulator formulation (BESF) on yield and accumulation of phenolic compounds in amaranth leaves is poorly understood. The current study assessed the effects of pre-sowing seed treatments with 0.0 %, 0.2 % and 0.4 % concentrations of BESF solution on germination, leaf yield, flavor and phenolic content in genetically selected *Amaranthus hybridus* var. *cruentus*. Data collected were subjected to analysis of variance (ANOVA). Significant treatment means were separated using Tukey test at $p \leq 0.05$. BESF significantly increased fresh marketable leaf yield by over 360 kg/ha (29 %) compared to the control. The total flavonoid content in the leaves was raised by 34 % and 47 % with 0.2 % and 0.4 % BESF solution treatments respectively, compared to control. Maximum concentration levels of rutin, apigenin, apigetrin, and quercetin was obtained with 0.4% BESF solution treatment. An analysis of the collected data suggest that BESF was effective in overall improvement in leaf yield, chemical content, and flavor of *A. hybridus* var. *cruentus*, allowing us to recommend BESF application to raise *A. hybridus* var. *cruentus* leaves for nutrition and pharmacological applications.

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1. Introduction

Amaranth (*Amaranthus spp*. L) (*Amaranthaceae*), an endemic plant of South and central America that now grows worldwide, is being cultivated in many temperate and tropical countries as a source of food, high quality forage and silage crop, and medicinal and ornamental applications [1- 4]. Although several species of amaranth are frequently considered weeds, the plant grows rapidly in hot weather conditions, accumulates concentrations of desirable

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bioactive constituents and is recognized as an effective food and medical source with macro- and micro- nutrients and other healthful bioactive compounds (secondary metabolites). In Africa, the plant is widely cultivated for leaf, under various agro-climatic and soil conditions during hot seasons, especially when common vegetables are scarce or difficult to locate [5, 6].

Thus, amaranth is promoted and increasingly consumed as leafy vegetable, and traditional medicine, where all parts of the plant are used as medicine to heal a number of human and animal diseases in most African communities [1, 2, 3, 5]. Freshly harvested amaranth leaf can be quickly boiled and consumed as a vegetable, mixed with locally grown spices along with fermented staple breads, such as wassa or kocho processed from enset (*Enset ventricosum*), tef (*Ergrostis tef*), sorghum, wheat, barley, legumes or root crops in most parts of Ethiopia [7]. With the population explosion from 25 million in 1977 to 100 million in 2016, there is an increasing challenge to satisfy the growing demand for fresh vegetables. This food need becomes overwhelmingly difficult and very limited in subsistent farming practices in highlands during long period of dry and hot seasons of the year.

The unfavorable climatic and ecological factors continue to reduce the growth, yield, and quality of active botanicals such as *A. hybridus* var *cruentus* despite the use of the latest achievements of genetic improvement, and modern plant growing technologies. Nigist [8] demonstrated the positive effects of urea and compost on growth and grain yield of *A. hybridus* var. *cruentus* in Ziway, Ethiopia. Onyango *et al*. [9] reported that the application of manure and mineral fertilizers improved seed germination, leaf yield, and mineral content of *A. hypochondriacus* in Kenya. It is important, however, to note that amaranth grown on land where chemical fertilizers are used or on nitrogen-rich soils, they accumulate nitrates and oxalate in the leaves, where nitrates are suspected to be implicated in stomach cancers, blue babies syndrome, and some other health problems [10-12], including being fatal to cattle in large quantities. It is desirable, therefore, to raise vegetable amaranth organically by using alternative method, such as use of BESF rather than chemical fertilizers.

Currently, recommended agronomic practices to improve the *A. hybridus* var *cruentus* leaf yield, nutritional quality, flavor, and its biochemical profile under organic cultivation do not exist. Furthermore, there has been no study to determine any induced response of *A. hybridus* var *cruentus* to pre-sowing seed treatments with BESF in respect to germination, leaf yield, flavor, and the biochemical contents in the leaves. BESF is a bio-transformed form of endophytic preparation from symbiotic endophytes (symbionts) of known medicinal plants origins, such as Echinacea. Though “symbiotic endophytes” may well be all microorganisms inhabiting plant organs while having a mutual symbiotic continuum ranging from mutualism to parasitism [13, 14] with possibility to transform to pathogenic [15, 16].

The main objective of our current study was, therefor, to evaluate the induced response of a genetically selected *A. hybridus* var. *cruentus* to pre-sowing seed treatment with BESF as a natural growth regulator, where we measured seed germination, morphology, leaf yield and flavor, and conducted lab tests for bioactive compounds. It is assumed that BESF will serve as an alternative to chemical fertilizers to raise vegetable amaranth that is, nutritious, healthy, and tasty.

2. Material and Methods

Amaranth (*A. hybridus* var. *cruentus*) seeds for the growth of plants in this study were purchased from a local farmer in Southern Ethiopia (ca 2100 m.a.s.l.), and subjected to a seed germination test to determine the seed viability [17]. Subsequently, the seeds were subjected to one of the following pre-sowing treatments for 12 h: Control (0.0 % pure water); 0.2 % and 0.4 % BESF plant growth regulator solution. The treated seeds were directly sown in organically farmed field of Gudar in southern Ethiopia during the rainy season (early July – mid September 2013). The leaves were harvested at maturity just before flowering and then weighed, boiled, and subjected to a flavor/taste test with a panel of 12 local people who were familiar with the plant.

2.1. Chemical analyses

Air dried leaves were weighed, ground, and extracted with 70 % ethanol (1 part leaf to 5 parts ethanol) for 24 h using continuous Soxhlet extractor apparatus. The ethanol extract was subsequently filtered and concentrated with a Büchi 461 rotary-evaporator (BUCHI Corporation, New Castle, Delaware, USA) at ca 40 °C.

Total phenolic contents (TPC) of the leaf extracts were measured using gallic acid, according to the method described by Singleton and Rossi [18], using Folin–Ciocalteau reagent with Na_2CO_3 (20%). The absorbance of the solutions was measured at 650 nm against the reagent used as a blank. A standard calibration plot was generated at 650 nm using known concentrations of gallic acid. The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as g of gallic acid equivalents (GAE) per 100 grams tissue dry-weight basis (g GAE/100 g dwt). Total flavonoid content in the extracts were measured according to the methods described by Zhishen *et al*. [19] and Mabry *et al*. [20], with the flavonoid contents expressed as quercetin equivalents (QE), and recorded as mg quercetin per g of dried extract.

2.2. Statistical analysis

Growth, yield, and biochemical tests were subjected to analysis of variance (ANOVA) using SPSS software version 12.0 (SPSS Inc. Chicago) with differences defined as $p \leq 0.05$. Regression analysis was used for assessment of a dose-response relationship. A probability value of $p \leq 0.05$ was considered significant.

3. Results

3.1. Agronomic traits

Pre-sowing seed treatment of *A. hybridus* with symbiotic growth regulator BESF solution positively affected the seed germination, seed emergence, plant height, leaf color, and marketable fresh leaf yield both at 0.2 % and 0.4% BESF solution compared to the control (Table 1). Pre-sowing seed treatment with BESF significantly ($p \leq 0.05$) influenced fresh edible leaf yield by over 360 kg/ha compared to the control (Table 1). Furthermore, the acceptance or likeness of taste and aroma (flavor) of steamed leaf of 0.4 % treatment received the highest (88 %) point by taste testing panel of 12 people compared to the control (Table 1). Increased plant height and leaf color, and numbers (data not shown) for 0.4% concentration of BESF treatment were significantly higher compared to the control (Table 1).

Table 1. Agronomic traits of Amaranthus hybridus var creuntus (L.) Thill as affected by different levels of pre-sowing seed treatment with symbiont BESF plant growth solution.

	Concentration levels of BESF solution		
Traits	0.0 % (control)	0.20%	0.40%
Seed germination (%)	89a	92a	97b
Seed emergence (%)	91a	94a	96b
Plant height at harvest (cm)	54a	56ab	60b
Fresh marketable leaf yield (kg/ha)	516a	764b	878c
Leaf flavor (taste), steamed (%)	74a	81b	88c
Leaf color at harvest	green	green	dark green

Note: Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

Table 2. Biochemical traits of *Amaranthus hybridus* var. *cruentus* (L.) Thill. as affected by pre-sowing seed treatment with different concentration of BESF solution.

	Concentration levels of BESF solution		
Chemical name	0.0 % (control)	0.20%	0.40%
Total polyphenols (PPs)	4.75a	5.87b	6.33c
Simple PPs & HBA[1]	0.60a	0.56a	0.58a
Hydroxycinnamic acid	0.22a	0.28a	0.21a
Condensed PPs	0.87a	0.93ab	1.04b
Total flavonoids	1.53a	2.05b	2.25b
Quercetin	0.32a	0.42b	0.44b
Rutin	0.42a	0.69b	0.72b
Apigenin	0.45a	0.53ab	0.61b
Apigenin-7-O-glucoside	0.34a	0.41b	0.48b

Note: Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

[1] Hydroxybenzoic acid

3.2. Biochemical traits

At eight weeks after sowing, the total concentration of polyphenols (PPs) in the leaves was 4.75 mg% in control, 5.8 mg% in 0.2%, and 6.33 mg% in 0.4% concentration of BESF solution growth regulator solution treatments, which were 24% and 33%, respectively, higher than the control ($p \leq 0.05$) (Table 2). Similarly, the condensed PPs content in the leaves rose from 0.87 mg% (control) to 1.04 mg% (0.4 % BESF solution concentration), which was about 20 % higher than the control ($p \leq 0.05$) (Table 2). The content of simple PPs, hydroxybenzoic acid (HBA), and hydroxycinnamic acid in the samples remained relatively stable under all treatments. In addition, the leaves from seed treatment at 0.2% and 0.4% concentration of BESF growth regulator solution, respectively, accumulated 34% and 47% more total flavonoids compared to leaves harvested from the control group ($p \leq 0.05$) (Table 2).

As shown in Table 2, other medically important plant constituents in the amaranth leaves were significantly increased in which seeds were pre-treated with BESF. On the average, the maximum concentration of rutin, apigenin, and apigenin-7-O-gluoside (apigetrin) and quercetin (Fig. 1) was obtained at 0.4% BESF solution, which enabled an increased concentration by 71%, 36%, 41% and 38%, respectively compared to the control ($p \leq 0.05$) (Table 2). It is important to note that BESF seed pretreatment showed the highest increase in rutin content. Rutin is among the flavonoids that has been reported to have many beneficial health effects, including that of a protective effect on memory dysfunction [21]. Our results on the response of leaf vegetable *A. hybridus*, var. *cruentus* is similar to the findings of Gins *et al*. [22] that applied pre-sowing seed treatment and foliar application of *Amaranthus tricolor*, L. with symbiotic microbial growth regulator. However, it is important and interesting to note, that, unlike our method that used Echinacea plants as source of the symbiotic microbial growth regulator, Gins *et al*. [22] applied different symbiotic growth regulators that were isolated from amaranth plants grown under totally contrasting ecological conditions.

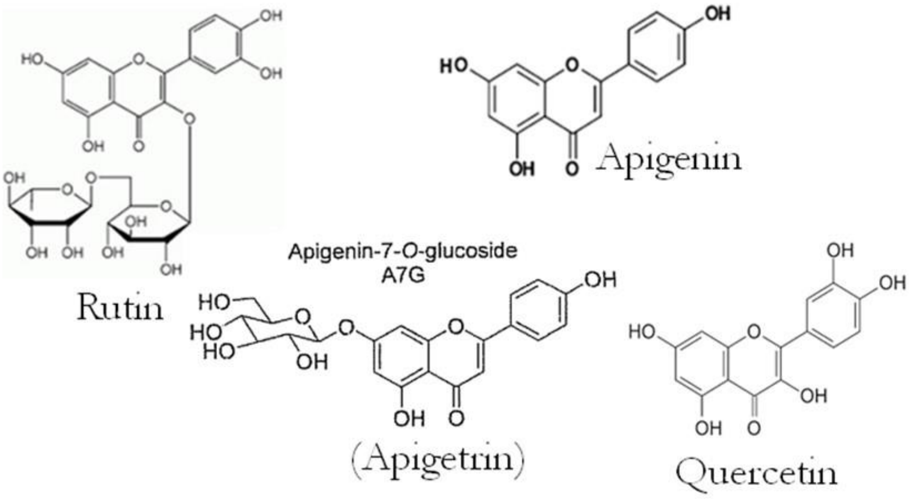

Figure 1. Biologically active constituents in vegetable *Amaranthus hybridus* var. *cruentus* (L.) Thill. leaves.

4. Discussion

Due to increasing global population with health consciousness, there is a growing demand for easily accessible, organically raised, leafy, healthful and nutritious vegetables in Africa, and elsewhere. *A. hybridus* var *cruentus* is one of those desirable popular alternatives growing widely under various agro-climatic conditions having less problems with pests and diseases. This is good news for subsistence farmers in Ethiopia, where there is limitations in rainfall, chemical fertilizers, pesticides, etc.

On the other hand, there is an increasing tendency to raise large fields of amaranth using chemical fertilizers to increase its yield [8, 9]. Amaranth grown under chemical fertilizers, urea, or nitrogen-rich soils tends to accumulate nitrates and oxalate in the leaves, where nitrate is suspected of causing stomach cancers, blue baby syndrome and some other health problems, including fatality to cattle in large quantities [10, 11, 12]. Fungal endophytes are known to occur in almost every plant species grown from the tropics to Antarctica and Arctic regions, having great potential in raising (cultivating) high quality plant products, including *in vitro* development and harvesting of pharmaceutical and nutraceutical products [23]. Endophytic symbionts can serve as elicitors of certain bioactive compounds, improve yield, and help protect plants from stressful conditions, such diseases, draught, etc. serving as alternative choice to pesticides or fungicides [24].

It has been shown that strains of symbiotic endophytes can be isolated from the host plant(s), where they can be cultivated under appropriate bioreactor to enable the endophytic bio-elicitors that enhance the root biomass and the concentration of pharmaceutically important compound, such as ginsenoside in ginseng adventitious roots [25]. Xiaolin *et al*. [25] reported the effects of endophytic bacterial elicitors on biomass and ginsenoside production in adventitious roots cultures of *Panax ginseng* C.A. Meyer (*Araliaceae*). Endophyte LB 5-3, as an elicitor, increased biomass and the content of total ginsenoside to 2.026 mg g^{-1} which was four times more than that in unchallenged roots (25). We did not determine the exact identity of the endophytic strain that we obtained from Echinacea. However, there is good evidence, from our experiment on *A. hybridus* var *cruentus*, that, symbionts can positively affect growth, yield and the content of healthful bioactive components in plants grown under unirrigated subsistence farming fields in southern Ethiopia highland. Hence endophytic symbiont (BESF) can be successfully used by subsistence farmers to produce high quality *A. hybridus* var *cruentus* as vegetable, with less or no nitrate, but higher content of bio-active secondary metabolites with flavorful taste. It is, however, important that locally identified and processed symbionts suitable for specific ecology must be developed and be available at an affordable price. For durable self-sufficiency, farmers need to be continuously trained or educated to enable them to responsibly practice sustainable, environmentally friendly agriculture and food production. Therefore, our program is focused to improve the challenges facing the rural agriculture, environment, and the human health by developing sustainable practices of agriculture and food production. In our present experiment, we have obtained promising results using a microbial symbiont BESF that increased leaf yield the bioactive chemical components with nutritional health and pharmacological applications. The symbiont BESF can be applied for organically raising valuable alternative vegetables, such as *A. hybridus* var *cruentus*.

5. Summary and Conclusion

Due to rapidly growing population and demand for fresh food, there is an increasing tendency to raise amaranth using major chemical fertilizers, such as nitrogen and phosphorus to increase the yield. Amaranth grown under chemical fertilizers or nitrogen-rich soils is known to concentrate nitrates and oxalate in the leaves. Nitrates are suspected to have caused health problems, such as stomach cancers, “blue baby syndrome” (methemoglobinemia) and fatal to cattle. In addition to being suspected of being contributors to the loss of soil microbial biodiversity and soil degradation, the cost of chemical fertilizers are steadily increasing as a consequence of the increasing energy prices. Hence, search for sustainable, environmentally friendly symbiotic microbial plant growth regulators is consistently increasing.

The main objective of our study was to evaluate the influence of pre-sowing seed treatment with bio-transformed fungal endophyte (symbiont) BESF on genetically selected *A. hybridus* var. *cruentus* raised in organically farmed field. We measured major traits, such as seed germination, morphology, leaf yield and flavor, and conducted biochemical tests in the lab for major bioactive compounds (secondary plant metabolites) of nutritional health value. Our research demonstrated that pre-sowing seed treatment of genetically selected *A. hybridus* var *cruentus* with BESF significantly improved agronomic traits, while enhancing the accumulation of bioactive constituents, such as apigenin, apigetrin, rutin, and quercetin compared to the unchallenged control. The blanched leaf flavor (palatability) as fresh vegetable was improved compared to the untreated plot. Our results show that BESF can be used successfully to grow vegetable *A. hybridus* var *cruentus* by subsistence farmers where they will be able to produce high quality plant material with no or less nitrates, to feed their families, domestic animals, and be able to market excess yield at premium prices in local markets. This study has shade some light on the promising prospects of using the symbiotic microbial growth elicitor BESF in one location, one growing season with one *A. hybridus* var *cruentus*. Therefore, we recommend further tests to elucidate the induced response of the selected variety to the application of microbial symbiont BESF under various environmental (growing) conditions, and cultivation practices in the country.

Conflict of Interst

None declared.

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