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Metabolic Fingerprinting of *Citrus* Cultivars and Related Genera Using HPLC and Multivariate Analysis

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Abstract

Citrus taxonomy is very complex and confusing, because of asexual reproduction and sexual compatibility between *Citrus* and related genera. Metabolic diversity was studied in *Citrus*, *Poncirus* and *Fortunella* cultivars by the high performance liquid chromatography technique combined with multivariate statistical analysis. Chromatograms obtained from cultivars of the same species showed similar elution profiles. These results suggested that metabolic profiles carry characteristics of hybrid origin. To confirm the similarities among the *Citrus* species and their cultivars, multivariate statistical analysis was applied to the chromatograms. According to hierarchical cluster analysis, all cultivars used in this study were divided into three major groups, which largely correspond to pummelo, mandarin and lemon. Hybrids were clustered together with their hybrid origin or their related cultivars. Our results indicated that the metabolic fingerprinting method provides an insight into the phylogenic relationships among *Citrus* species and cultivars.

Keywords: chemotaxonomy, *Citrus*, metabolic fingerprinting, secondary metabolite

1. Introduction

The true citrus fruit trees comprise six genera, i.e. *Fortunella, Poncirus, Citrus, Microcitrus, Eremocitrus* and *Clymenia.* Among these, the *Citrus* species is one of the most commercially important groups and has a wide diversity of related cultivars. However, *Citrus* taxonomy and phylogeny are very complex and confusing because of asexual seed reproduction and sexual compatibility between *Citrus* and related genera. Until the mid 1970s, studies on *Citrus* taxonomy were carried out based on morphological and geographical data, and numbers of classification systems were formulated. Among them, the classification systems formulated by Swingle (1943) and Tanaka (1969) have been widely accepted. However, these two widely accepted systems are based on different classification concepts. Swingle identified only 16 species, while Tanaka recognized 162 species. Later, Scora (1975) and Barret and Rhodes (1976) proposed that there are only three species in the subgenus *Citrus*, i.e. citron (*Citrus medica* L.), mandarin (*Citrus reticulata* Blanco) and pummelo (*Citrus grandis* (L.) Osbeck), and the other genotypes have originated by hybridization between these three true species. Recently, this three-species concept has been supported by many studies using biochemical and molecular markers, such as isozymes (Torres *et al*., 1978; Fang *et al*., 1993; Herrero *et al*., 1996), microsatellites (Fang and Roose, 1997; Fang *et al*., 1998) and nuclear (Ramadugu *et al*., 2013) and organellar genome analyses (Green *et al*., 1986; Yamamoto *et al*., 1993; Nicolosi *et al*., 2000; Carbonell-Caballero *et al*., 2015).

Since a variety of secondary metabolites present in higher plants apparently serve as defense compounds against environmental stresses, they are important for plant survival. Thus, the distribution of secondary metabolites appears to represent the adaptive characters that have been evolved through natural selection. Since secondary metabolites within the members of a family are often similar, their distribution of secondary metabolites could be used as one of the markers for phylogenetic and taxonomic classifications.

Early studies on *Citrus* chemotaxonomy based on secondary metabolites, were carried out using thin-layer chromatography (Albach and Redman, 1969; Tatum *et al*., 1974) and paper chromatography techniques (Dass *et al*., 1977, 1978; Grieve and Scora, 1980; Handa, 1988). These methods are effective for comparing patterns of pigment composition, but require visual estimation of the separated compounds for their quantification. Recently, high-performance liquid chromatography (HPLC) has been used for the separation and quantification of metabolites, and multivariate statistical methods have also been introduced for estimating similarities in chemical composition. Gayou *et al*. (1987) analyzed six polymethoxylated flavones in peel oils of six *Citrus*

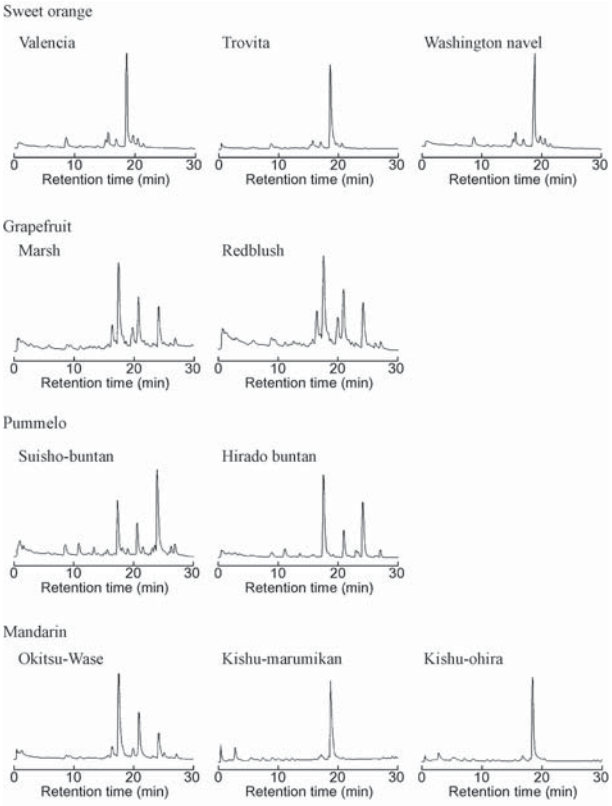
cultivars and applied a multivariate statistical analysis to sample classification. Kawaii *et al*. (1999) also quantitatively identified 24 flavonoids in fruits in order to determine the relationships among 66 *Citrus* species.

These studies focused on fruit metabolites because of their commercial importance. However, since leaves are a richer source of secondary metabolites in higher plants, metabolites in leaves should also be included in chemotaxonomic analysis. The previous authors purified metabolites, identified their chemical structures and determined their amounts. However, the requirement for the preparation of authentic compounds and baseline separation on HPLC limits the analysis to a narrow range of metabolites, and thus, restricsts their application for taxonomic analysis of a wide range of cultivars and species. Recently, metabolomic approaches using LC/MS and GC/MS techniques have been used for qualitative analysis (Sumner et al., 2003). These techniques have become powerful tool to gain comprehensive information of metabolites in plant materials. However, these techniques need expensive devises and specific software which handle a large amount of information produced by the devices. Moreover, since identification of metabolites depends on mass spectra databases, application of these techniques is limited to primary metabolites or secondary metabolites of model organisms. In order to provide the relationships between the different species based on secondary metabolites, we performed a comprehensive analysis of UV absorbing secondary metabolites such as flavonoids and coumarins, major compounds in citrus, in leaves by using conventional HPLC, and the elution profiles were applied to chromatographic pattern analysis based on multivariate statistical analysis.

2. Materials and Methods

2.1 Plant Material

The youngest and fully expanded leaves of 29 cultivars (Table 1) were harvested and collected from mature trees maintained in Yuasa Experimental Farm, Wakayama Prefecture, Japan. For minimize the environmental condition, all trees used in this study were grown in the same fields. Five biological replicates were collected for each species.



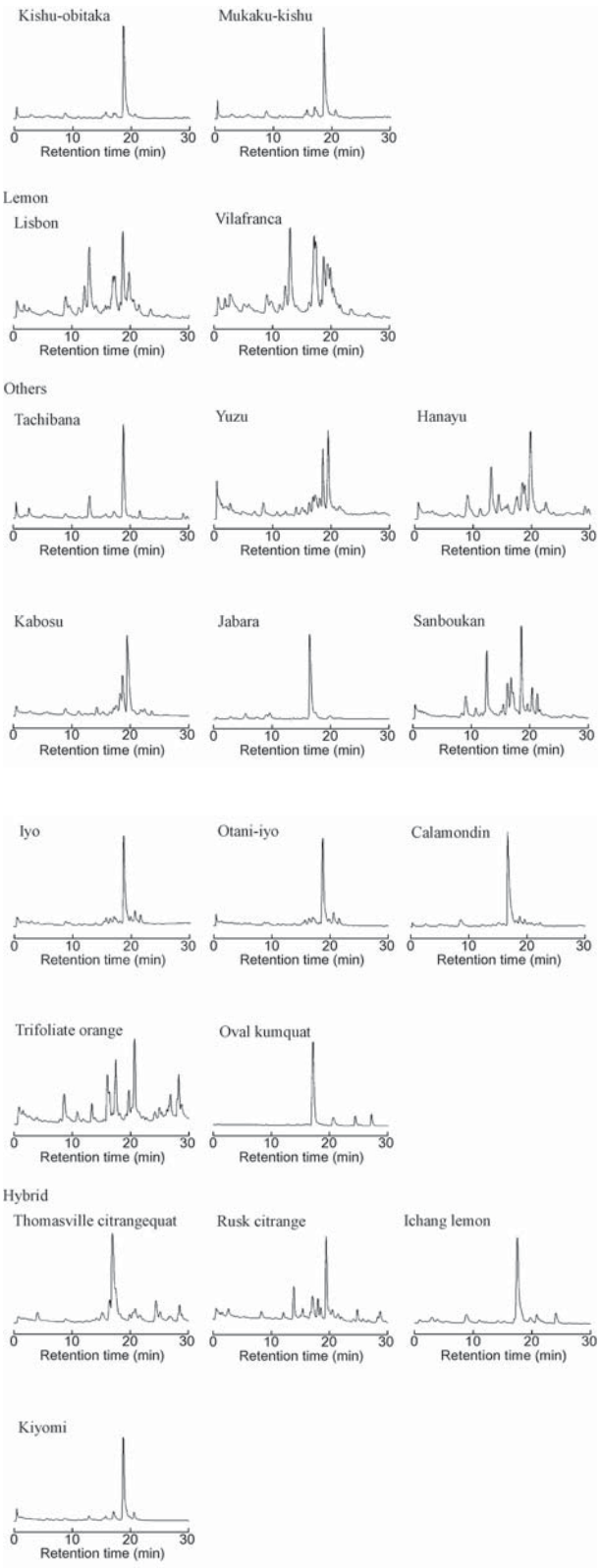


Figure 1. HPLC chromatograms of MeOH extracts obtained from mature leaves of *Citrus* and related genera. Elution of metabolites was monitored at 280 nm

Table 1. Plant materials used in this study

	Scientific name[a]	
Cultivar	Swingle's system	Tanaka's system
Sweet orange	*Citrus sinensis* (L.) Osbeck	*Citrus sinensis* (L.) Osbeck
Trovita		
Valencia		
Washington		
Navel		
Grapefruit	*C. paradisi* Macf.	*C. paradisi* Macf.
Marsh		
Redblush		
Pummelo	*C. grandis* (L.) Osbeck	*C. grandis* (L.) Osbeck
Suisho buntan		
Hirado buntan		
Mandarin		
Okitsu Wase	*C. reticulata* Blanco	*C. unshiu* Marc.
Kishu	-[b]	*C. kinokuni* Hort. ex. Tan.
marumikan		
Kishu ohira	-	*C. kinokuni* Hort. ex. Tan.
Kishu obitaka	-	*C. kinokuni* Hort. ex. Tan.
Mukaku kishu	-	*C. kinokuni* Hort. ex. Tan.
Lemon	*C. limon* Osbeck	*C. limon* Osbeck
Lisbon		
Villafranca		
Others		
Tachibana	-	*C. tachibana* (Mak.) Tan.
Yuzu	-	*C. junos* Sieb. ex. Tan.
Hanayu	-	*C. hanayu* Sieb. ex. Tan.
Kabosu	*C. aurantium*	*C. sphaernocarp* Hort. ex. Y. Tan.
Jabara	-	*C. jabara* Hort. ex. Y. Tanaka
Samboukan	-	*C. sulcata* Hort. ex. Takahashi
Iyo	-	*C. iyo* Hort. ex. Tan.
Ohtani iyo	-	*C. iyo* Hort. ex. Tan.
Calamondin	-	*C. madurensis* Lour.
'Oval' kumquat	*Fortunella margarita* Swingle	*Fortunella margarita* Swingle
Trifoliate orange	*Poncirus trifoliata* Raf.	*Poncirus trifoliata* Raf.
Hybrid		
Thomasville	*F. margarita* × (*P. trifoliata* × *C. sinensis*)	
Rusk	*C. sinensis* × *P. trifoliata*	
Ichang lemon	*C. ichangensis* × *C. grandis*	
Kiyomi	*C. unshiu* × *C. sinensis*	

[a] Botanical names are based on Swingle's system. In addition to this system, the botanical names of Tanaka's system are mentioned for species indigenous to Japan.

[b] not identified

2.2 Extraction of Secondary Metabolites

Citrus leaves were frozen in liquid nitrogen and ground to a fine powder. The powder was extracted with 5 volumes of methanol, a suitable solvent for extraction of semi-polar metabolites (Ballester *et al*., 2016), and centrifuged at 12,000 *g* for 15 min. An aliquot of the supernatant was filtered, and the filtrate was subjected to HPLC analysis.

2.3 HPLC Condition

The extract was analyzed by an Shimadzu 10A system (Shimadzu, Kyoto, Japan) with a 4.6 i.d. × 50 mm Cosmosil 3C18 AR-II column (Nacalai tesque, Kyoto, Japan). A two solvent system was used to generate the mobile phase—solvent A was 0.1% acetic acid solution and solvent B was MeOH. The flow rate was 1.2 ml/min.

The mobile phase at the beginning of the analysis was 18% B in A. After the injection of the extract, the ratio was maintained for 5 min, followed by a linear gradient from 18% to 38% B in A in 15 min. Subsequently, a 10 min linear gradient from 38% to 58% B in A was applied. The elution of secondary metabolites was monitored at 286 nm.

2.4 Statistical Analysis

To apply a multivariate analysis to the chromatogram profile, raw chromatograms obtained from each run were scanned and transformed to digital (x, y) data by a digitizing program Un-Scan-It (Silk Scientific, Inc., UT). All the transformed data were imported to the multivariate data analysis program Pirouette 3.11 (Infometrix, Inc., WA). The elution profiles of five replicates were averaged, and the differences in the elution time of each run were adjusted and aligned with reference to the co-chromatogram data of each sample. The aligned data set was used for hierarchical cluster analysis (HCA). The multivariate measure of the distance between each cluster was based on Euclidean distance, and the cluster was linked based on the incremental method.

3. Results and Discussion

3.1 HPLC Chromatograms

All the leaf samples used in this study were harvested from the same field on the same day, and thus, were grown under the same climatic conditions. The leaf samples were extracted with methanol and subjected to reverse phase HPLC analyses. The applied gradient system covers the major UV active compounds, including flavonoids, coumarins and phenylpropanoids.

As shown in Figure. 1, the chromatograms obtained from cultivars of the same species showed largely similar elution profiles. In the chromatograms of sweet orange (*C. sinensis*) cultivars, all the major peaks were common to the cultivars and were of a similar size. Almost all peaks were common to the two cultivars of grapefruit (*C. paradisi*), and in pummelos (*C. grandis*), the three major peaks detected in 'Suisho buntan' were also detected in 'Hirado buntan'. In contrast, mandarin cultivars showed heterogeneity in the elution profiles. 'Okitsu Wase' showed three major peaks, which were not detected in the other mandarin cultivars. On the other hand, the largest peak commonly detected in 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' was absent in 'Okitsu Wase'. 'Okitsu Wase' are considered to belong to satsuma mandarins, while others are kishu mandarins, and thus, the differences in elution profiles reflect the difference between satsuma and kishu. In the chromatograms of the two lemon (*C. limon*) cultivars, many peaks overlapped each other. The similarity between the elution profiles of the two cultivars was observed, although the intensity of each peaks was different among two species.

On comparing the elution profiles of the species, a similarity was detected between the elution profiles of grapefruit and pummelo cultivars. The retention times of the three major peaks in the grapefruit cultivars were completely consistent with those of the two pummelo cultivars, although the relative sizes of the peaks in the two species were different. The elution profile of the mandarin cultivar 'Okitsu Wase' (*C. unshiu*) also resembled those of the other species.

The elution profile of 'Kiyomi' was quite similar to those of the sweet orange cultivars. 'Kiyomi' is the artificial hybrid of 'Miyagawa Wase' (*C. unshiu*) and 'Trovita' (*C. sinensis*) (Nishiura *et al*., 1983). Thus, it was found that the metabolic character of 'Trovita' was dominantly inherited through the screening and breeding program.

Other cultivars showed cultivar-specific chromatograms. Many of these cultivars are thought to be indigenous to Japan and their origin is still obscure. Among them, cultivars classified as *C. iyo* ('Iyo' and 'Ohtani iyo') showed similar elution profiles. Their profiles were also similar to those of the four mandarin cultivars 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu', suggesting that these two groups might have originated from the same ancestor.

3.2 Hierarchical Cluster Analysis

To confirm the similarities among the *Citrus* species and their cultivars, the elution profiles were subjected to the hierarchical cluster analysis. Chromatograms were converted to 1000 data points, and the data was clustered by the application of an incremental linkage method based on Euclidean distance. Figure. 2 shows the dendrogram constructed by using the hierarchical cluster analysis.

Based on this dendrogram, the analyzed cultivars were divided into three major groups. The first group contained three cultivars of sweet orange (*C. sinensis*), and the four mandarin cultivars namely, 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' (*C. kinokuni*). In addition, the two *C. iyo* cultivars, 'Iyo' and 'Ohtani iyo' as well as the other *Citrus* cultivars 'Tachibana' (*C. tachibana*), 'Kabosu' (*C.*

sphaernocarp), 'Yuzu' (*C. junos*), 'Hanayu' (*C. hanayu*), 'Calamondin' (*C. madurensis*) and the two hybrids 'Kiyomi' and 'Rusk' citrange were included in this group. The second group consisted mainly of the grapefruit and pummelo cultivars. 'Oval' kumquat (*F. margarita*) and trifoliate orange (*C. trifoliata*) were also clustered in the second group. The third group contained cultivars of lemon (*C. limon*) and 'Jabara' (*C. jabara*). Barret and Rhodes (1976) postulated that the diverse genotypes in the genus *Citrus* originated from only three species, namely, mandarin (*C. reticulata*), pummelo (*C. grandis*) and citron (*C. medica*). The three groups observed in our dendrogram largely corresponded to the mandarin, pummelo and citron groups, supporting the three species concept (Scora, 1975; Barret and Rhodes, 1976; Nicolosi *et al*., 2000; Carbonell-Caballero *et al*., 2015).

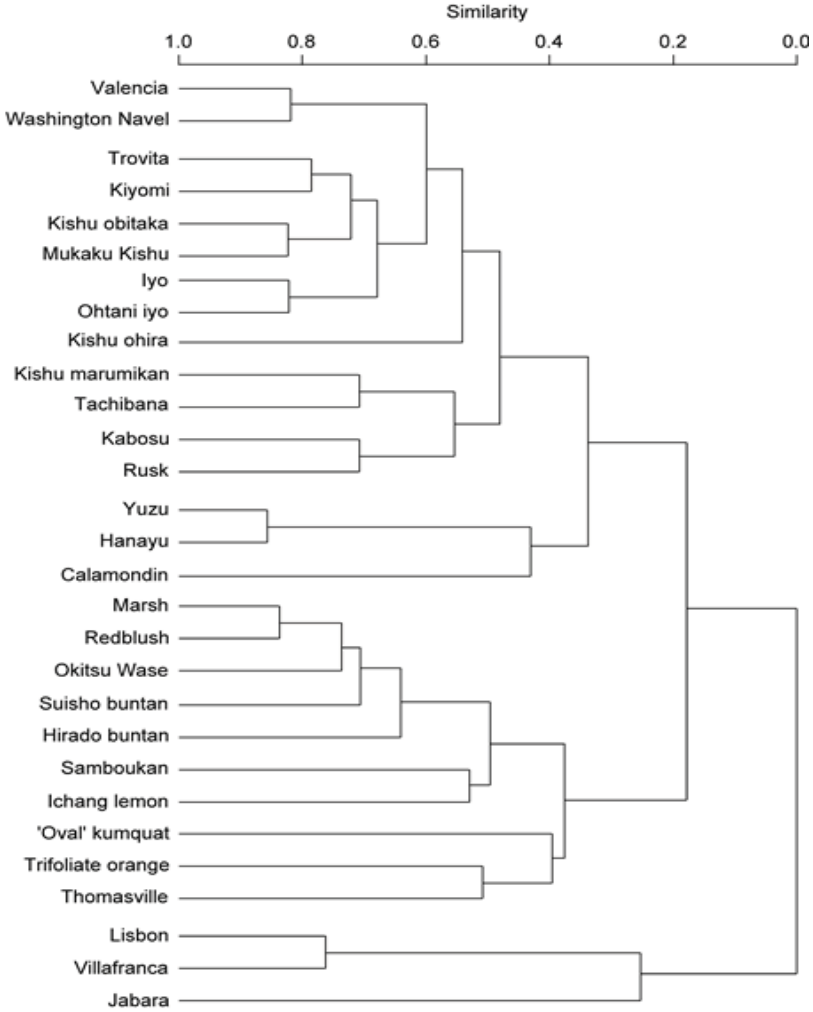


Figure 2. Dendrogram of 29 cultivars of *Citrus* and related genera obtained by hierarchical cluster analysis of HPLC chromatogram data

The first group was divided into two subgroups; the first subgroup was composed mainly of sweet orange cultivars and four mandarin cultivars, and the other subgroup was composed of the cultivars 'Yuzu', 'Hanayu' and 'Calamondin'. 'Iyo' and 'Ohtani iyo' were included in the first subgroup together with the sweet orange cultivars. Since it has been suggested that 'Iyo' and 'Ohtani iyo' are natural tangors (Tanaka, 1954), their close relationship with sweet oranges is reasonable. 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' (*C. kinokuni*) were also clustered in the first subgroup. The phylogenic relationship between *C. kinokuni* and the other cultivars is unclear. The close relationship between *C. kinokuni* and *C. sinensis* suggests that *C. reticulata* was involved in the differentiation of *C. kinokuni* because *C. sinensis* have been thought to be of predominantly the *C. reticulata* genotype introgressed with genes from *C. grandis* (Barrett and Rhodes, 1976; Nicolosi *et al*., 2000). Moreover, *C. sinensis* is now thought to originate from backcross hybrid between pummelo and mandarin (Xu *et al*., 2013). The phylogenic relationship between 'Tachibana' and 'Kabosu' and sweet oranges may suggest the involvement of *C. reticulata* in the differentiation of these cultivars. 'Yuzu',

'Hanayu' and 'Calamondin' were clustered as the second subgroup in the first group, suggesting the introgression of traits of *C. reticulata* into the cultivars.

'Kiyomi' was closely related to orange cultivars, such as 'Valencia', 'Trovita' and 'Washington Navel'. 'Kiyomi' is the artificial hybrid of 'Miyagawa Wase' unshu (*C. unshiu*) and 'Trovita' orange (*C. sinensis*) (Nishiura *et al*., 1983). Thus, the clustering of 'Kiyomi' with 'Trovita' reflects the origin of 'Kiyomi' and the metabolic characteristics of 'Trovita' may be predominantly inherited in this hybrid. Among sweet orange cultivars 'Kiyomi' with 'Trovita' showed the closest relationship, which indicates the utility of the hierarchical cluster analysis of elution profiles of the *Citrus* cultivars.

The second group consists of 11 cultivars and is divided into two subgroups. The first subgroup includes grapefruit (*C. paradisi*) cultivars, the mandarin cultivar 'Okitsu Wase' (*C. unshiu*), pummelo (*C. grandis*) cultivars, 'Ichang lemon' (*C. ichangensis* × unknown cultivar of *C. grandis*) and 'Samboukan' (*C. sulcata*). It has been suggested that grapefruit is derived from a sweet orange and pummelo hybrid (Scora, 1975; Barrett and Rhodes, 1976; Scora *et al*., 1982 Scora and Kumamoto, 1983; Nicolosi *et al*., 2000). The strong similarity between grapefruit cultivars and those of pummelo was indicated in our analysis, whereas no similarity was observed between grapefruit and sweet oranges. This is consistent with previous studies that indicate the close relationship between grapefruit and pummelo (Swingle and Reece, 1967; Handa, 1988). The close similarity between satsuma mandarin (*C. unshiu*) and pummelos and grapefruits was an unexpected result. Satsuma mandarin is believed to have originated in Japan as a chance seedling from a fruit or to have been imported from China (Swingle and Reece, 1967). Swingle (1943) identified it as one of the *C. reticulata* hybrids, while Tanaka (1977) recognized it as one of the *Citrus* species. In this study, metabolic patterns and statistical analysis showed a high similarity between satsuma mandarin and *C. grandis*, suggesting that some pummelo-type species were involved in the origin of satsuma mandarin. Tanaka (1932) named Ichang lemon *C. wilsonii* and stated that this species unquestionably had *C. junos* as one parent and that it appeared to be a cultigen that had originated as a chance seedling. On the contrary, Swingle and Reece (1967) stated that there was no valid reason for considering this plant as a botanical species since it is undoubtedly a chance hybrid of *C. ichangensis*. Based on our results, the characteristics of *C. grandis* might be strongly appeared. 'Samboukan' is indigenous to Japan, and its origin and its related species or cultivars are unknown. Our results suggest that pummelos were involved in the origin or differentiation of this cultivar.

The second subgroup contains 'Oval' kumquat (*F. margarita*), trifoliate orange (*P. trifoliata*) and 'Thomasville' citrangequat. However, 'Oval' kumquat cluster showed low similarity to the *Poncirus* cluster. 'Thomasville' citrangequat is an artificial trigeneric hybrid of 'Oval' kumquat (*F. margarita*) and 'Wiltis' citrange (*P. trifoliata* × *C. sinensis*). Our results indicate that the metabolic profile of 'Thomasville' citrangequat is closer to *P. trifoliata*, suggesting that the metabolic character of *P. trifoliata* is dominantly inherited.

The last group is composed of lemon cultivars (*C. limon*) and 'Jabara' (*C. jabara*). Based on morphological characteristics, lemon was supposed to be a hybrid of citron (*C. medica*) and lime (*C. aurantifolia*) (Swingle, 1943; Malik *et al*., 1974; Scora, 1975). Barrett and Rhodes (1976) reported that lemon is a trihybrid of citron, pummelo and *Microcitrus* and had a higher proportion of citron genes. Torres *et al*. (1978) suggested that sour orange (*C. aurantium*) and lime are of hybrid origin. Based on the molecular marker data, Yamamoto *et al*. (1993) suggested that lemon is a hybrid of pummelo and citron, while Nicolosi *et al*. (2000) reported that lemon originated from citron and lime. Other report suggested that lemon originated from the complex hybridization of *C. maxima, C. reticulata* and *C. medica* (Clurk *et al*., 2014). Lemon cultivars clustered separately from the mandarin and pummelo clusters because of the strong influence of citron. 'Jabara' is indigenous to Japan and is cultivated in a limited area. Its origin is obscure and is thought to be a cultivar related to 'Yuzu' or 'Kunembo' mandarin. In order to determine the origin of this unique cultivar, the analysis of lime and citron will be necessary.

In summary, our chemotaxonomic results were broadly in accordance with the three-species concept. Although our method cannot provide the structural information of individual peaks, the method using conventional HPLC combined with multivariate statistical analysis was proven to be a simple and powerful tool for providing new insights into the phylogenic relationship among the *Citrus* cultivars and hybrids. Several questions and unexpected results have to be investigated. Further studies with a large number of cultivars and species would provide us with a deeper understanding of *Citrus* phylogeny and taxonomy.

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In vitro and *in vivo* Assay for Assessment of the Biological Control Potential of *Streptomyces* sp. KT

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Abstract

Rhizoctonia solani is fungal plant pathogen that infects many different host plants. Recently, biological control agents that are friendly to the environment and ecosystems have attracted much attention as an alternative to the use of chemical fungicide which have been used worldwide to control soil borne pathogens including *R. solani*. In this study, 53 strains of actinomycetes isolated from environmental soils, and antifungal activities of them were assessed by the dual culture assay. Strain KT showed strong inhibitory activities against 8 phytopathogenic fungi. A great suppressive effect on *R. solani* growth was observed in the inoculation test of plants using cucumber and chin-geng-sai. In addition, infection of *Bipolaris oryzae* also could be suppressed in the detached leaf assay using oats. As a result of genetic analysis, it was shown that KT was a species closely related to *Streptomyces lavenduligriseus* NRRL B-3173^T. However, as far as we know, there is no report for biological control agents using *S. lavenduligriseus*. This study suggests that the strain KT may useful as biological control agents to suppress various crop diseases.

Keywords: biological control, actinomycetes, plant pathogens

1. Introduction

Currently the world population is increasing more than 7 billion people and is projected to reach over 9.8 billion people by 2050 (United Nations, 2017). On the other hand, the arable land area per capita in the world continues to decrease, and it is predicted that in 2050 it will decrease to 30% compared to 1950 (FAO, 2012). Therefore, improvements of the crop yields must be necessary in limited agricultural land. In order to contribute to solve this problems, reducing the loss of crops in the cultivation steps is important. Plant pathogens are one of the main causes of losses of crop production and pesticides are an effective means to prevent losses in crop production.

Rhizoctonia solani is a very influential plant pathogen in crop production and infects many different host plants, causing damping-off, sheath blight, stem canker, and seed decay. As the disease of *Rhizoctonia* occurs in all parts of the world, many researches have been done since the discovery of *R. solani* in 1858 (Ogoshi, 1987). In case of rice, yield losses due to sheath blight disease caused by this pathogen was estimated to range from 5 to 10% in Asia. Moreover, the estimated yield losses increased up to 50% when susceptible cultivars were grown under suitable conditions for this pathogen. (Lee & Rush, 1983, Marchetti & Bollich, 1991, Savary, Willocquet, Elazegui, Castilla, & Teng, 2000). In potato, *R. solani* causes stem canker in some case, and marketable yield losses have been reported to be approaching 30% (Banville, 1989). In the case of cucumber fields, the average annual loss in the United States is approximately 7-9%, which equals to a loss of approximately 4-5 million dollars (Lewis & Papavizas, 1980). In recent years, up to 75% mortality in cucumber seedlings in the most affected greenhouse has been reported in countries where greenhouse cultivation is major agriculture (Al-Sa'di, Drenth, Deadman, De Cock, and Aitken, 2007). Therefore, cucumber damping-off is a serious problem not only in agricultural field but also in greenhouse cultivation (Al-Sa'di et al, 2007, Lewis & Lumsden, 2001). In Japan, it has been reported that *R. solani* K1 isolated in Kanagawa prefecture in 1986 causes stem rot in various crops such as rice, eggplant, cucumber, spinach, tomato (Asaka & Shoda, 1996). From these facts, controlling plant pathogens, especially controlling *R. solani* is an important issue for improving agricultural production.

Chemical fungicides have been used worldwide to control soil borne pathogens including *R. solani* (Parry, 1990). However, chemical fungicide application has some problems, such as environmental pollution, deteriorating

human health, and development of drug-resistant pathogens/insects (Clevo & Clem, 2001). Furthermore, current chemical agents are not completely effective and *Rhizoctonia* disease remains to be a persistent problem. Therefore, biological control agents that are friendly to the environment and ecosystems have attracted much attention as an alternative to the use of chemical fungicide.

In recent years, biocontrol of soil born disease, particularly using antibiotic activity of actinomycetes, has been put forward as an alternative to chemical control agents. Actinomycetes are filamentous soil bacteria and also known for their ability to produce many antibacterial/antifungal compounds (Sanglier, Haag, Huck and Fehr, 1996, Jiayue et al., 2016). They also secrete extracellular hydrolytic enzymes such as chitinases and glucanases, which can degrade components in the cell walls of plant pathogens (Castillo et al., 2016, Sakdapetsiri et al., 2016). Therefore, actinomycetes are promising group of root-colonizing microbes where they may influence plants by promoting growth and protecting them from diseases. However, there have been a few reports under unsterilized condition, a similar condition at open field. The objective of this study is to isolate an actinomycete that has a strong antifungal activity and bring out their potential ability to suppress the diseases caused by *R. solani* and other phytopathogens.

2. Method

2.1 Microorganisms

Actinomycete strain KT was isolated from the soil sample collected in Wakayama, Japan and incubated on PDA (PDA; BD Difco) at 24 °C for 7 days. Isolated colonies of the actinomycetes were preserved in glycerol suspension (15% v/v) as a stock at -30 °C. The plant pathogenic fungi [(*Bipolaris oryzae* MAFF 305382, *Colletotrichum echinochloae* MAFF 305460, *Colletotrichum orbiculare* MAFF 306685, *Fusarium oxysporum* MAFF 103038, *Fusarium solani* MAFF 235170, *Monilinia fructigena* MAFF 305640, *Phytophthora infestans* MAFF 236324, *Rhizoctonia solani* MAFF 235846), *Rhizoctonia solani* K-1] were incubated on potato dextrose agar (PDA) at 24°C for 7 days before use.

2.2 Identification of Actinomycete Strain KT

Phylogenetic analysis based on 16S rDNA sequence and morphological observation were done as follows. Bacterial 16S rRNA genes were PCR-amplified with primers 9F (5'-GAGTTTGATCCTGGCTCAG) and 1510R (5'-GGCTACCTTGTTACGA). The 16S rRNA gene sequences determined were compared with those retrieved from DNA database of APORON DB-BA 11.0 (Techno Suruga Lab., Shizuoka, Japan) and GenBank/EMBL/DDBJ using a BLAST homology search, and phylogenetic tree was constructed to ascertain the phylogenetic position of the actinomycete strain KT. Phylogenetic trees were generated by the neighbor-joining method. Gene sequencing and phylogenetic analysis were carried out at Techno Suruga Lab., Co., Ltd. (Shizuoka, Japan).

2.3 Dual Culture Assay for in vitro Inhibition of Mycelial Growth of Plant Pathogens

Antifungal activity of KT against plant pathogens was evaluated by using dual assay. An agar plug of 0.7-cm diameter from actively growing fungal mycelia was placed on the perimeters of the PDA plate at a distance of 1.5 cm, and then strain KT was inoculated on the opposite of the fungal mycelia. Plates were incubated for 7-14 days at 24°C. The zone of inhibition was measured as halo area around the actinomycete strain KT, and inhibition index was determined by the following criteria; +: <20 cm^2, ++:20-30 cm^2, +++: >30 cm^2.

2.4 Infection Control of Isolated Actinomycete Strain KT against R. solani

Cucumis sativus L. (cucumber) and *Brassica rapa* var. *chinensis* (qing-geng-cai) were used for infection control test. Seeds were soaked in 70% ethanol for 10 sec, and sterilized with 1%NaClO for 10 min, finally rinsed three times with sterile distilled water. Sterilized seeds were germinated under dark conditions for 3 days on water agar plate. The germinated seeds were then transferred to agripot containing modified Hyponex medium (Hyponex powder; 0.0375%, sucrose; 0.35%, agar; 0.8%, pH6.0), with 3 (cucumber) or 5 (qing-geng-cai) seeds in one pot. KT was cultured in PDB medium at 24˚C, 1500 rpm (stirred culture) for 3 days and 200 μL of the culture broth was spread in the agripot on the day of transplant. Five days after the transplant, the infection assay was carried out by placing 0.7-cm-diameter agar plugs of plant pathogens onto the center of agripots. Biocontrol effect was assessed after the plants were grown in growth chamber for 15-20 days at 28˚C, 12 h light/dark condition.

Seed and soil were not sterilized in the soil experiment, and 2 g of wet hyphae of KT were used in the experiment. Disease severity was evaluated according to the 0–5 indexes (0: no disease, 1: browning of root, 2: browning of stem, 3: wilting of plants, 4: lodging of plants, 5: plant death), and determined by the following equation;

$$\text{Disease index} = \frac{\Sigma(\text{score of disease} * \text{number of plants})}{\text{total number of plants}}$$

2.5 Detached Leaf Assay of Isolated Actinomycete Strain KT against B. oryzae

Avena sativa (oat) was used for detached leaf assay. Plants at 2 to 3 true leaf stage in pots were harvested and washed with distilled water. They were cut into uniform lengths of 1.5 cm and cut edge was sealed with paraffin. Sealed leaves were sterilized with 1%NaClO for 90 sec and rinsed three times with sterile distilled water. After sterilized, leaves were soaked in KT culture for 10 min. Then, leaves were placed in 12-well microplate with sterilized moistened gauze. For the detached leaf assay, mycelial plug (4 mm^2) of *Bipolaris oryzae* from PDA medium was inoculated onto the upper surface of the *Avena* leaves pierced with needle of syringe followed by the incubation at 25 °C, 12-h light/dark conditions for 12 days. Disease severity was evaluated according to the leaf area covered by the fungus by the following 0–4 indexes (0: no disease, 1: hyphal formation around the agar pieces, 2: hyphal coverage of less than 50%, 3: hyphal coverage of 50-80%, 4: completely covered with hyphe).

3. Results

3.1 Dual Culture Assay for in Vitro Inhibition of Mycelial Growth of Plant Pathogens

Fifty-three actinomycete strains were isolated from environmental soil samples, and anti-fungal activities of them were assessed by the dual culture assay using *R. solani*. Among them, an isolate named KT showed strong suppressive effects on the growth of *R. solani*. The inhibitory effect for other phytopathogens was assessed in the same way as *R. solani*. Antifungal activity of the strain KT was assessed by measuring the inhibition zones present after incubation of dual culture with 8 fungi (Fig. 1). The strong antagonistic activities (+++) against *B. oryzae*, *C. echinochloae*, *C. orbiculare* and *F. oxysporum* were observed in the dual culture plates, and the inhibition zones were 42.1, 30.9, 37.5, 31.6 cm^2, respectively (Fig. 1 and Table 1). KT also showed moderate antagonistic activity (+ and ++) against other pathogenic fungi.

3.2 Identification of Actinomycete Strain KT

The actinomycete strain KT was isolated from a soil sample on the root of a tree collected at Wakayama, Japan. KT showed the filamentous growth and was gram-positive. It formed 2.0-3.0 mm white colony and aerial hyphae, but, did not form spores. The 16S rDNA sequence of actinomycete strain KT shows high sequence similarity to genus *Streptomyces*, and the closest similarity with that of *S. lavenduligriseus* NRRL B-3173^T (Accession No. NR043824) with 99.6% similarity (Fig.2).

3.3 Infection Control of Rhizoctonia Damping-off of Cucumber by Strain KT

As shown in Fig.3B, symptom of *R. solani* infection was observed on the third day after inoculation in the control experiment, and severe damage was observed from days 6 to 8. But in the KT treated pots, only a weak infection was observed as shown in Figs. 3A, B. Since the clear suppression of *R. solani* growth was observed in cucumber which belongs to Cucurbitaceae, we performed the same assay using other plant species, qing-geng-cai in the family of Brassicaceae, in order to reveal whether the effect depends on host plants. The first infection was observed on the day 7 in the control experiment, and it was 4 days later compared with that of cucumber, but the severe damage was observed from days 15 to 20. However, in the KT treated experiment, strong suppressive effect against the infection by *R. solani* was clearly observed (Figs. 4A, B). Since the remarkable inhibitory effect by KT against the infection of *R. solani* was observed in the Hyponex medium, a sterilized and nutrition-rich medium, the similar experiment was done using the unsterilized soil, which is similar to the actual field. As shown in Figs. 5,6, KT showed a noticeable inhibitory effect against the infection by *R. solani* and protected cucumber and qing-geng-cai from the infection even in the non-sterile soil.

3.4 Detached Leaf Assay of Isolated Actinomycete Strain KT against B. oryzae

As the clear suppressive effect against *R. solani* was observed, another important phytopathogenic fungus *B. oryzae*, which causes disease symptoms mainly in high value field crops in the family Poaceae, including rice, maize, wheat, was used. To assess whether actinomycete KT could control the plant pathogen *B. oryzae*, *A. sativa* (oat) was used for detached leaf assay. Oat leaves were treated with or without KT. It was observed that leaves with soaking treatment of KT culture inhibited the growth of the plant pathogen and showed almost no lesion on the leaves, while the leaves of non-treated plants were covered by the pathogens as show in Fig.7A (top panels). The KT-treated leaves showed little mycelial formation of *B. oryzae* even on the final day of the test, and the disease severity was also low (Fig.7B).

4. Discussion

There are a variety of microorganisms in the rhizosphere of plants. Interaction between plants and microorganisms influence on the plant growth in a positive or negative manner. Biological control agents are thought to be able to promote the growth of plants and suppress plant pathogens. Actinomycete is one of the potential biological control agents for plant disease and they are known to produce useful antimicrobial compounds.

Growth of 8 plant pathogens was inhibited in vitro assay by dual culture method, and strain KT had the strongest antifungal activity in the actinomycetes that were collected in our library. These results suggested the presence of antifungal metabolites which control wide range of plant pathogens. In this study, a novel strain *Streptomyces* sp. KT was evaluated as a biological control agent against cucumber damping-off, qing-geng-cai damping-off caused by *R. solani* and oat leaf blight caused by *B. oryzae*.

Disease suppressive potential of KT was tested by cucumber or qing-geng-cai infection control assay. The strain KT was able to protect the seedlings of cucumber or qing-geng-cai from *R. solani*. Infection control tests were performed on both Hyponex medium and unsterilized soil. Both tests showed successful suppression against plant pathogens. Previous studies often use culture medium and sterilized soil, but few reports use unsterilized soil. The experiments using unsterilized soil cannot avoid the influence of microorganisms present in the unsterilized soil, and thus, the focused microorganism is difficult to proliferate. However, KT could inhibit the growth of phytopathogenic fungi under the unsterilized soil condition. This result suggests that pretreatment with KT culture can reduce the infection of a plant pathogen even in the open fields.

In this study, we tried infection control tests on not only *Rhizoctonia* damping-off but also Brown spot (*B. oryzae*). *B. oryzae* is known to cause brown spot in the Poaceae family, and brown spot is diseases that cause important losses between 1-10%. (Sarvary et al., 2000, 2006) Disease area of brown spot is the third largest in Japan, following blast rice blight (neck rot) and sheath blight (Matsumoto et al., 2016). Control of brown spot is an important issue for the rice cultivation area. KT can significantly delay the infection of *B. oryzae* in the detached leaf assay. Therefore, KT may be useful as a biological control agent to manage many plant pathogens in view of the results of dual culture assay.

The results of phylogenetic analysis based on 16S rDNA sequences indicate that actinomycete strain KT belongs to the cluster of the genus *Streptomyces*, however, the strain KT showed different phylogenetic position with *S. lavenduligriseus* NRRL B-3173^T. Differences of 6 base in the 16S rDNA base sequence was observed between *S. lavenduligriseus* NRRL B-3173^T and strain KT. Among the difference of the segment, 2 bases are suggested to be caused by insertion/deletion, and the remaining 4 bases was clearly different. From these results, KT was identified as *Streptomyces* sp., which closely related to strain *S. lavenduligriseus* NRRL B-3173^T. *S. lavenduligriseus* is known to produces pentenomycin and polyene macrolides (Jiayue et al., 2016). Pentenomycin is antibacterial compound and is not effective against fungi. On the other hand, polyene macrolides show a broad antifungal spectrum, and inhibition of plant pathogens has been reported, which may suggest that the antifungal compound of KT has also a similar structure (Kim et al., 2012). As far as we know, there is no report for biological control agents using *S. lavenduligriseus*. Therefore, research on the use of strain KT is considered to contribute to the development of novel biological control agents in the future.

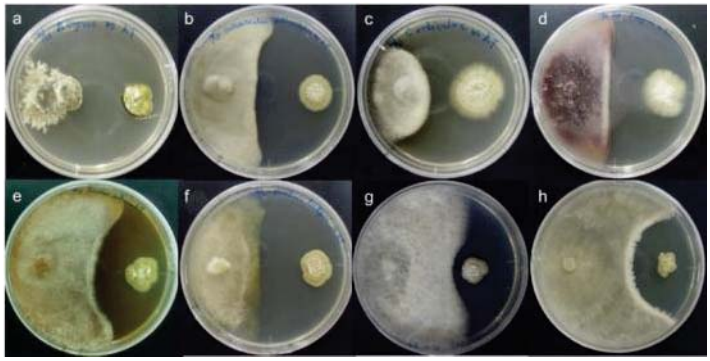

Figure 1. Dual culture assay of actinomycete strain KT (right) against plant pathogens (left). Strain KT and plant pathogens were inoculated on the PDA medium at the same time. (a) *Bipolaris oryzae*, (b) *Colletotrichum echinochloae*, (c) *Colletotrichum orbiculare*, (d) *Fusarium oxysporum*, (e) *Fusarium solani*, (f) *Monilinia fructigena*, (g) *Phytophthora infestans*, (h) *Rhizoctonia solani*

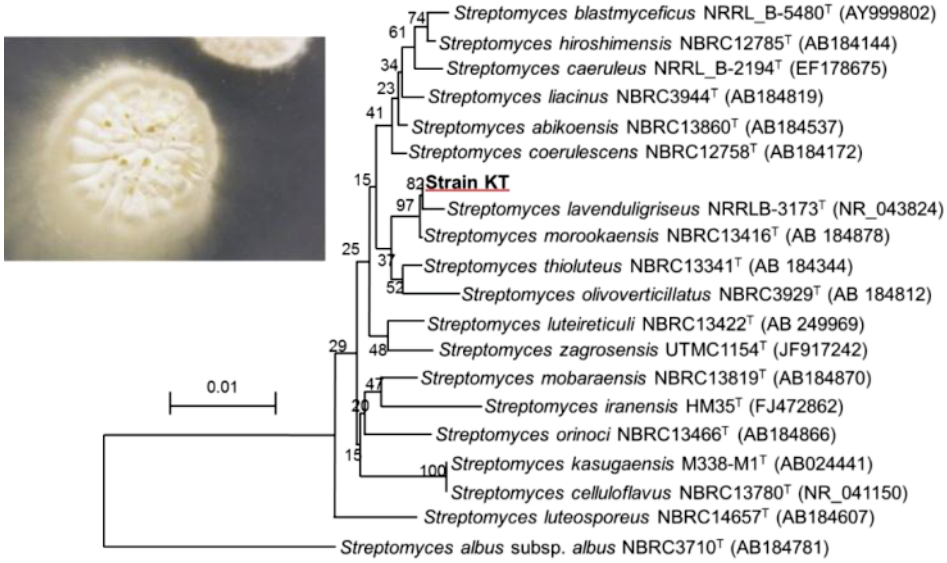

Figure 2. 16S rDNA gene sequences showing relationships between *Streptomyces* isolates and related *Streptomyces* type strain species. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets

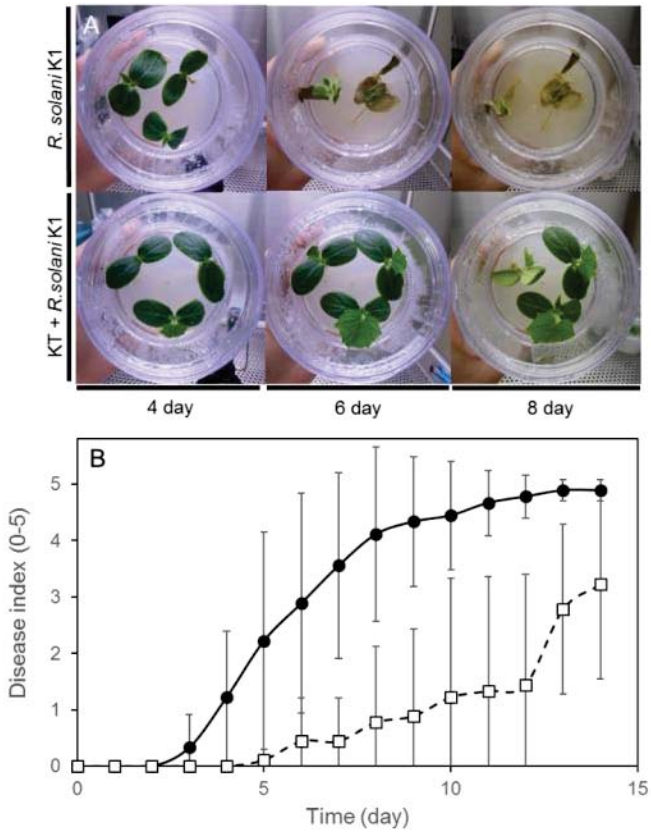

Figure 3. Infection control of strain KT against cucumber damping-off (*R. solani*). The photograph of infection control of *R. solani* by strain KT(A). Reduction of disease severity of cucumber damping-off by strain KT(B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

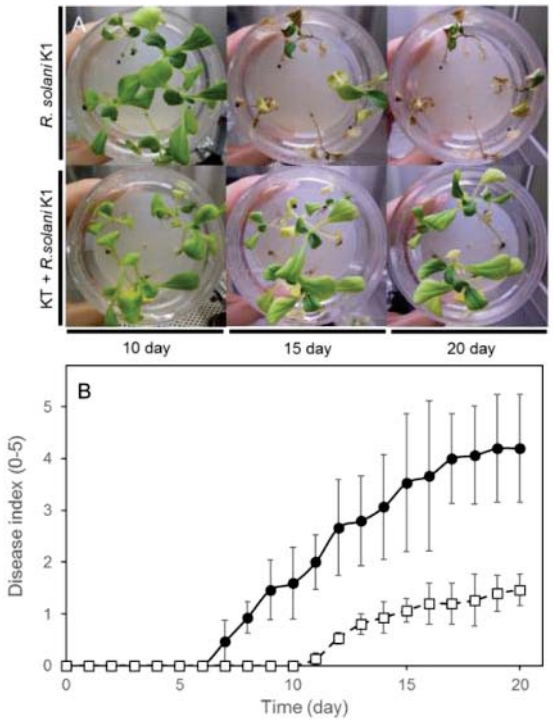

Figure 4. Infection control of strain KT against qing-geng-cai damping-off *(R. solani*). The photograph of infection control of *R. solani* by strain KT(A). Reduction of disease severity of qing-geng-cai damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

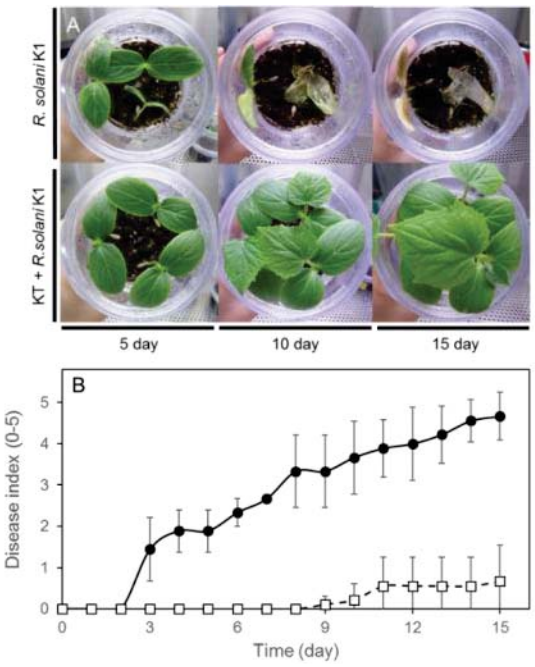

Figure 5. Infection control of strain KT against cucumber damping-off (*R. solani*) under unsterilized soil condition. The photograph of infection control of *R. solani* by strain KT (A). Reduction of disease severity of cucumber damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

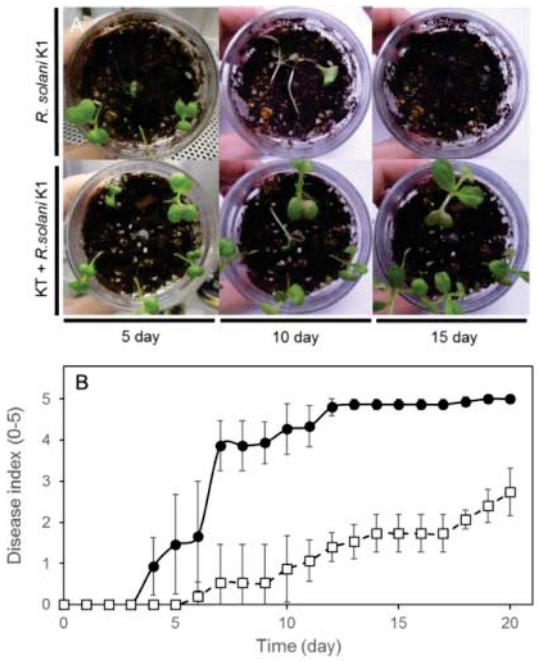

Figure 6. Infection control of strain KT against qing-geng-cai damping-off (*R. solani*) under unsterilized soil condition. The photograph of infection control of *R. solani* by strain KT (A). Reduction of disease severity of qing-geng-cai damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

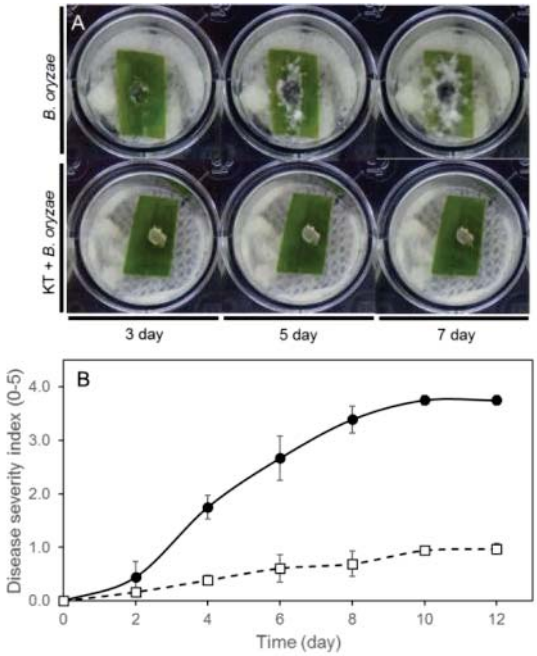

Figure 7. Detached leaf assay of strain KT against *Bipolaris* leaf blight. The top panels showed the result inoculated with *B. oryzae* and bottom panels showed that inoculated with *B. oryzae* and strain KT (A). Reduction of disease severity of Oat leaf blight (*B. oryzae*) by strain KT was clearly observed (B). Inoculated with only *B. oryzae* (closed circle). Inoculated with *B. oryzae* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-4 value (B). The same experiment was repeated three times, and the average value and standard deviation were shown

Table 1. Antifungal activity of actinomycete strain KT against plant pathogens on PDA.

Plant pathogens	Crops	Inhibition zone area (cm^2)	Inhibition index*
Bipolaris oryzae	rice	42.1±0.6	+++
Colletotrichum echinochloae	Japanese millet	30.9±0.5	+++
Colletotrichum orbiculare	watermelon	37.5±0.8	+++
Fusarium oxysporum	cabbage	31.6±0.5	+++
Fusarium solani	rice	18.6±0.1	+
Monilinia fructigena	apple	29.6±0.5	++
Phytophthora infestans	tomato	14.1±0.2	+
Rhizoctonia solani	lawn grass	11.7±0.2	+

(*inhibition zone +, < 20 cm^2; ++, 20 - 30 cm^2; +++, > 30 cm^2)

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A Review of Medicinal Uses and Pharmacological Activities of *Tridax Procumbens (L.)*

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Abstract

Tridax procumbens is a very promising species that produces secondary metabolites reported to have a variety of medicinal uses including among others, anti-anemic, anti-inflammatory, anti-diabetic and anesthetic properties. This species has a long history of traditional use by different communities. This study aimed to review the scientific literature regarding the medicinal properties, biological activity and phytochemical components of *T. procumbens*, a member of the Asteraceae family that originated in Central and South America. An extensive literature review was done using Metadatabase EDS, MedLine (PubMed), Science Direct, Web of Science, Academic Search Premier, Scielo, DOAJ Directory of Open Access Journals, JSTOR, and other sources to find information relevant to the medicinal uses of *T. procumbens*. At total of 130 studies were found that contained information about *T. procumbens*. Some of the papers were not included because of the relevance to this study, ending with a total of 111 relevant citations reported here. This review shows the importance of more studies to understand the potential of *T. procumbens'* secondary metabolites for medicinal or preventive treatment, making it a promising ethnobotanical resource. This review provides important information of this species and indicates that this species could be an effective, safe and affordable treatment for some ailments, especially in tropical areas where this plant is native and widely distributed.

Keywords: *Tridax procumbens*, anti-inflammatory, anti-diabetic, immunomodulatory, antimicrobial, hepatoprotection, anti-hypertensive

1. Introduction

Tridax procumbens, also known as "coat buttons" is a perennial plant from the Asteraceae family, native to Central and South America (Hilliard, 1977; Ravikumar et al., 2005b). Since ancient times, this species has been used in Ayurveda in India (Kethamakka and Deogade, 2014). Different substances such as oils, teas and skin poultices, among others, have been manufactured using this species (Foret, 2012). *T. procumbens* has diverse pharmacological properties including but not limited to: immunomodulatory, anti-oxidant, anti-hepatotoxic, analgesic, antidiabetic, anti-inflammatory, antifungal, and antimicrobial activities. (Ravikumar et al., 2005a; Ravikumar et al., 2005b; Bhagwat et al., 2008; Sawant et al., 2014; Hitesh, 2006). The versatility of the species is most likely due to the plant's defense mechanisms, secondary metabolites such as flavonoids, alkaloids, tannins, carotenoids and saponins. The aim of this review is to highlight the importance of this species as a valuable medicinal plant. The connection of the traditional and scientific knowledge is important for future studies.

1.1 Botanical Description

Tridax procumbens (family Asteraceae) is known by different names throughout the world (Table 1).

Table 1. Common names of T. procumbens found throughout the world.

Country/ Language	Vernacular Names	Source
Chinese	Kotobukigiku	Ankita and Jain 2012
English	Coat buttons, Tridax daisy	USDA, Ankita and Jain 2012, Kumar et al., 2012; Chauhan and Johnson, 2008; Ravikumar et al., 2005b, Bhagwat et al., 2008.
French	Herbe Caille	Ankita and Jain 2012
Latin	*Tridax procumbens (Linn.)*	Ankita and Jain 2012
Malayalam	Chiravanak	Ankita and Jain 2012
Marathi	Dagadi Pala	Ankita and Jain 2012
Oriya	Bishalya Karani	Ankita and Jain 2012
Sanskrit	Jayanti Veda	Ankita and Jain 2012
Spanish	Cadillo, Chisaca	ITIS, ND, Ankita and Jain 2012
Telugu	Gaddi Chemanthi	Ankita and Jain 2012
Tamil	Thata poodu	Ankita and Jain 2012
Australia	Tridax daisy	Holm et al., 1997
Brazil	Erva de Touro	Holm et al., 1997
Burma	Mive Sok Ne-gya	Holm et al., 1997
Burundi	Agatabi	Byavu et al., 2000
Colombia	Cadillo Chisaca	Holm et al., 1997
Cuba	Romerillo de Loma, Romerillo	Holm et al., 1997
Dominican Republic	Piquant Jambe	Holm et al., 1997
El Salvador	Hierba del Toro	Holm et al., 1997
Fiji	Wild Daisy	Holm et al., 1997
Ghana	White-dirty Cream, Nantwi bini	Holm et al., 1997; Komlaga et al., 2015
Guatemala	Bull Grass, Bull's herb	Vibrans 2009, Gamboa-Leon et al., 2014
Hawaii	Tridax	Holm et al., 1997
Honduras	Hierba del Toro	Holm et al., 1997
India	Bisalyakarmi, Mukkuthipoo, Phanafuli, Tunki, Ghamara, Javanti Veda, Dhaman grass, Vettukkayapoondu, Vettu kaaya	Holm et al., 1997; Kumar et al., 2012; Kethamakka and Deogade, 2014; Pareek et al., 2009; Ravikumar et al., 2005b, Bhagwat et al., 2008, Silambarasan and Ayyanar, 2015, Yabesh et al., 2014.
Indonesia	Gletang, Gletangan, Sidowlo, Tar Sentaran	Holm et al., 1997
Jamaica	Bakenbox	Mitchell and Ahmad, 2006
Japan	Kotobukigiku	Holm et al., 1997
Java	Songgolangit	Petchi et al., 2013
Madagascar	Anganiay	Holm et al., 1997
Malaysia	Coat Buttons, Kanching Baju	Holm et al., 1997
Mauritius	Herbe Caille	Holm et al., 1997
Mexico	Flor Amarilla, Panquica, Rosilla, *t'ulum*	Holm et al., 1997, Gamboa-Leon et al., 2014
Nigeria	Igbalobe, Muwagun, Muriyam pachila, Jayanti, Vettukkaaya-thala	Olowokudejo et al., 2008; Soladoye et al., 2013, Sureshkumar et al., 2017
Puerto Rico	Tridax	Holm et al., 1997
Taiwan	Kotobuki-giku	Holm et al., 1997
Thailand	Teen Tuk Kae	Holm et al., 1997
Trinidad	Railway Weed	Holm et al., 1997
United States	Tridax daisy	Holm et al., 1997

T. procumbens is found in tropical and subtropical areas of the world growing with annual crops, along roadsides, pastures, fallow land, and waste areas (Holm et al., 1997). The species has a diploid number of 36 (Raghavan and Vinkatusabban, 1941). It has herbaceous, semi-prostrate habit, and can grow anywhere from 15-40 cm in height. The leaves are elongated, opposite, ovate with serrated margins, hirsute on the abaxial and adaxial sides (Powell, 1965). The inflorescence is a capitulum with three-toothed white ligulate ray florets female and disc inner flowers yellow, tubular, bisexual, with corolla 6 mm long. The inflorescence results in abundant production of pappus achenes (Chauha and Johnson, 2008), 2 mm long, obovoid, setaceous, covered with stiff hairs, that can be carried by the wind for long distances, making this species a potential invasive species if not controlled.

T. procumbens is classified as a noxious weed in Alabama, Florida, Minnesota, North and South Carolina and Vermont. It is quarantined in California and Oregon and prohibited in Massachusetts (U. S. Department of

Agriculture). In Guatemala *T. procumbens* is a weed that has a wide range of growth and can be found in either dry or damp soil, usually on previously cultivated ground from sea level to 2300 m (Pöll, 2005).

2. Traditional Uses

Traditional and complementary medicine is being increasingly recognized as an integrative approach to health care in many countries (WHO, 2013). The use of plants for medicinal purposes may date back to the Middle Paleolithic age, approximately 60,000 years ago (Solecki, 1975). *T. procumbens* is found throughout the world (Table 2) and it has been used to treat anemia, colds, inflammation, and hepatopathies in Central America (Taddei and Rosas-Romero, 2000). In Guatemala, *T. procumbens* is used as an antibacterial, antifungal, and antiviral treatment (Caceres et al., 1998) as well as for vaginitis, stomach pain, diarrhea, mucosal inflammations, and skin infections (Taddei and Rosas-Romero, 2000). The leaf juice is used to treat wounds and stop bleeding (Caceres et al., 1998). A study done in Chiquimula, Guatemala, showed that lactating pregnant women suffering from anemia could reduce their symptoms by using *Tridax* (Calderón, unpublished results). This species is also used in the treatment of gastrointestinal and respiratory infections, high blood pressure, and diabetes (Pöll, 2005, Giovannini et al., 2016. Pardeshi and Bhiungade, 2016). In Guatemala, the entire plant is used for the treatment of protozoal infections (Caceres et al., 1998; Berger et al., 1998, Martín-Quintal et al., 2009, Gamboa-Leon et al., 2014, Ebiloma et al., 2017), including malaria, leishmaniasis and dysentery. Aqueous extracts of *T. procumbens* have strong anti-plasmodial activity against chloroquine-resistant *P. falciparum* parasites (Appiah-Opong et al., 2011); it has activity against *Trypanosoma brucei*, antibacterial and wound-healing properties (Koram et al., 2014, Agyare et al., 2016). Scientific support for several of these traditional uses will be discussed later.

Table 2. Traditional uses and plant preparation

Location		Preparation/extract	Plant ailment uses	References
Guatemala		Leaves: juice	Anemia, colds, inflammation, hepatopathies, vaginitis, stomach pain, diarrhea, mucosal inflammation, skin infections, bleeding.	Caceres et al., 1998; Taddei and Rosas-Romero, 2000
		Leaves: poultice, dried infusions Stems: dried	Reduce inflammation, gastrointestinal and respiratory infections, high blood pressure, diabetes	Pöll, 2005, Giovannini et al., 2016
		Whole plant: dried	Protozoal infections, treatment of chronic ulcers caused by leishmaniasis, gastrointestinal disorders	Berger et al., 1998. Martín-Quintal et al., 2009; Gamboa-Leon et al., 2014 Ebiloma et al., 2017
India		Leaves: dried and other herbs ingested orally, juice	Diabetes, insect repellent, used to treat diarrhea, and to help check for hemorrhages, as well as hair loss. Jaundice, healing of wounds, inflammation	Pareek et al., 2009, Policegoudra et al., 2014; Saraf et al., 1990, Saraf and Dixit, 1991, Rajendran et al., 2003, Taddei and Rosas-Romero, 2000, Yabesth et al., 2014; Pardeshi and Bhiungade, 2016.
Africa		Whole plant: blending with other herbs adding salt and water	Treating mastitis in livestock	Byavu et al., 2000
Africa	Ghana	Decoction with *Phyllanthus amarus*	Anti-malarial, antibacterial, wound-healing	Koram et al., 2014
		Aqueous extracts	Anti-plasmodial activity	Appiah-Opong et al., 2011, Komlaga et al., 2015
	Nigeria	Whole plant: dried	Fever, Typhoid fever, cough, back ache, stomach ache, diarrhea, epilepsy	Soladoye et al., 2013. Mann et al., 2003
	Benin	Whole plant: dried	Rabbit or livestock feed	Aboh et al., 2002, Edeoga et al., 2005
	Togo	Leaves: dried	Dressing wounds, pain, malaria and abdominal and gastrointestinal mycosis	Agban et al., 2013

In Nigeria, the entire plant is used to treat typhoid fever, cough, fever, stomachache, backache, diarrhea and epilepsy (Soladoye et al., 2013; Mann et al., 2003). Farmers in Africa use the plant for treatment of livestock (Byavu et al., 2000); for example, *Tridax* is used along *Vigna parkeri* to treat chronic mastitis by grinding both plants, adding salt and water and applying to the udder. Ayyappa Das et al. (2009) studied the antibacterial effect of *Tridax* against mastitis-causing bacteria and found that the ethanolic extract had significant activity against *Staphylococcus aureus*. However, there was little or no activity from the aqueous extracts against *Streptococcus uberis* and *Klebsiella penumonia*, in comparison with *Spathodea campanulata* extracts. In Benin, breeders complement the feed of rabbits (Aboh et al., 2002) or other livestock combining with other plants (Edeoga et al., 2005); although rabbits consume it in lower amounts than other fodder (Aboh et al., 2002), probably due to low

palatability.

In Togo, the fresh, crushed leaves are used for dressing wounds. The decoction of the leaves is used against pain, to treat malaria, and against abdominal and gastrointestinal mycosis (Agban et al., 2013). In India it is known as an insect repellent, used to treat diarrhea, and to help check for hemorrhages. In addition, some reports include the use as a cure for hair loss (Policegoudra et al., 2014; Saraf et al., 1990) and jaundice (Saraf and Dixit, 1991).

A study in Tamilnadu, India, revealed that native inhabitants apply the juice from the leaves for the healing of wounds. The same study also infers that *T. procumbens* is one of the most useful traditional medicinal plants (Rajendran et al., 2003). It has also been shown to have many minerals like calcium, selenium, magnesium, potassium and sodium (Ikewuchi et al., 2009). The people in Udaipur, India, have traditionally ingested powdered *T. procumbens* leaves, along with other herbs, to treat diabetes (Pareek et al., 2009; Pardeshi and Bhiungade, 2016). The species has shown to be a great source of potassium, which is used for the treatment of cramps and a safe source ingredient for future medicinal uses. These traditional uses (Table 2) demonstrate the potential uses of this plant.

3. Phytochemistry

T. procumbens use as a traditional medicine throughout various regions of the world has led to many publications on its phytochemistry (Table 3). The discovery of new bioactive compounds can lead to the development of new drugs for the treatment of various ailments (Fabricant and Farnsworth 2001). Different extraction techniques used to isolate various compounds found in *T. procumbens* will be discussed.

Table 3. Phytochemicals found in *Tridax procumbens*

Extraction	Compounds/activity	Plant organ	References
Aqueous	Antidiabetic compounds	Aerial parts	Caceres et al., 1998 Ikewuchi, 2012.
Chloroform, Acetone	Tannins,condensed catechic	Leaves	Sawant and Godhate 2013
Ethyl acetate, aqueous, ethanol	Flavonoids, kaempferol, (-)-Epicatechin, Isoquercetin, and Glucoluteolin	Leaves, Stem, Root, and Flowers	Kumar et al., 2012; Harborne, 1994.
Aqueous	Alkaloids, Akuammide and Vaucangine	Leaves.	Ikewuchi 2012.
Methanol- dichloromethane	Bioactive components for antifungal activity against dermatophytes.	Aerial parts.	Policegoudra et al., 2014.
Ethanol- acetic acid	Alkaloids for antimicrobial activity, against human pathogens, antioxidant, Hepatoprotective	Pedicle and buds.	Jindal and Kumar 2012. Hemalatha 2008.
Petroleum Ether	Antioxidant uses against DPPH.	Dried plants.	Saxena et al., 1977.
Distilled Water- ethanol	Immuno-modulatory effects in rats.	Aerial parts.	Tiwari et al., 2004
methanol -n-butanol	Isolation of antioxidant chemicals, mostly flavonoids and saponins	Dried leaves.	Saxena et al., 2013
methanol-ethyl acetate	Isolation of antioxidant chemicals for testing: mostly Flavonoids and saponins.	Dried leaves.	Saxena et al., 2013
n-hexane	Antimicrobial against *Mycobacterium smegmatis,Escherichia coli, Salmonella* spp.	Flowers and aerial parts.	Kethamakka and Deogade, 2014.
Ethanol	Saponin B-Sitosterol-3-O-β-D-xylopyranoside.	Flowers	Saxena and Albert, 2004
Petroleum ether, ethanol	Anti-ulcerogenic effects	Leaves	Jhariya et al., 2015
Hydro-distillation	Essential oil, anti-microbial and anti-inflammatory effects. Terpenes, alpha and beta pinenes	Leaves.	Manjamalai et al., 2012b
Ethanolic extract	Phytochemical screening: alkaloids, glycosides	Whole plant dried.	Kamble and Dahake, 2015

3.1 Phytochemical Screening

Many studies have been done on the phytochemistry of Tridax, given the potential of this species (Tables 3 and 4), resulting in a variety of compounds. For example, anthraquinones, anthrones, flavonoids, and steroids are found in leaves in relative abundance (Nisha, 2011). The secondary metabolites that contain medicinal properties are discussed throughout this paper, showing the importance of these extraction methods. Although the compounds have been identified, the exact bioactive compounds responsible for the medicinal properties are still unknown. Many of the compounds identified have unknown metabolic pathways and a variety of bioactive

compounds may work in conjunction to elicit medicinal properties.

3.2 Primary Metabolites

Primary metabolites involved in metabolic pathways present in all plants. There are a few specific primary metabolites that have been extracted from *T. procumbens:* Lipids are essential in living organisms; they influence the communication between cells, the cellular makeup, and act as an energy source for the organism. *T. procumbens* contains common fats found in the Asteraceae family. This species also exhibits some lipids that give the plant unique properties and promising medicinal uses. These unique fats have been extracted and include: methyl 14-oxooctadecanoate, methyl 14 oxononacosanoate, 3-methylnonadecylbenzene, heptacosanyl cyclohexane carboxylate, 1(2,2-dimethyl-3-hydroxypropyl)-2-isobutyl phthalate, 12-hydroxytetracosan-15-one, 32-methyl-30-oxotetratriacont-31-en-1-ol and 30-methyl-28-oxodotriacont-29-en-1-oic acid dotriacontanol, β-amyrone, Δ^{12}-dehydrolupen-3-one, β-amyrin, lupeol, fucosterol, 9-oxoheptadecane, 10-oxononadecane and sitosterol (Verma and Gupta, 1988). All these compounds play essential roles in plants and are common to many species.

3.3 Secondary Metabolites

Secondary metabolites are compounds produced by plants that are not essential for the normal growth and development of the plant, but play an important role in plant defenses, communication, stress responses and others. Secondary metabolites contain bioactive compounds that often have useful and important medicinal properties. Some of the most important bioactive compounds for medicinal uses are found in compounds such as glycosides, nitrogenous organic compounds, fat-soluble compounds, polyphenolic compounds, and minerals (Edeoga et al., 2005). *T. procumbens* secondary metabolites have been included into six major groups: flavonoids, carotenoids, alkaloids, saponins, tannins, and terpenes.

3.3.1 Flavonoids

Flavonoids are found in the leaves and other organs (Jhariya et al., 2015) and haves shown to be useful as anticoagulants, hair tonics, anti-fungal, against problems of bronchial catarrh, diarrhea, dysentery, and wound healing (Ali et al., 2001). The presence of procumbenetin and other flavonoids in *Tridax* seem to decrease the deposition of calcium and oxalate in the kidneys (Sailaja et al., 2012). This secondary metabolite seems to help regenerate damaged beta cells of the pancreas (Petchi et al., 2013). Evaluation of an aqueous extract of *T. procumbens* for its effect on diabetic rats showed hypoglycemic activity (assumed from flavonoids), protection against oxidative stress (probably due to high content of ascorbic acid) and lowering of VLDL cholesterol (probably due to the flavonoids) (Ikewuchi, 2012).

Luteolin and Quercetin were also isolated from *Tridax*, along with the flavonoid Procumbenetin (Jhariya et al., 2015). Lutein, glucoluteolin, and isoquercetin are found in the flowers of *T. procumbens* (Kumar et al., 2012). Luteolin has anti-inflammatory and anti-carcinogenic activity (Rao et al., 2012), probably due to its anti-oxidant activity and its free-radical scavenging ability (Seelinger et al., 2008). Luteolin has shown strong inhibition of tumor proliferation by suppressing angiogenesis (Kawaii et al., 1999). In vitro studies indicate that Luteolin has activity against different cancer cell lines including breast cancer (Tu et al., 2013), liver cancer (Pettit et al., 1996), hepatoma (Chang et al., 2005), colon cancer (Leung et al., 2006), human lung squamous carcinoma (Leung et al., 2005) and uterine cancer (Makino et al., 1998). In vivo studies have also shown anti-carcinogenic activity of Luteolin; for example, immunodeficient SCID mice and nude mice with prostate adenocarcinoma (Chiu and Lin, 2008; Markaverich et al., 1997; Fang et al., 2007) showed reduction in the size of the tumors when treated with Luteolin. Luteolin seems to slow the migration and invasion of cancer cells (Lin et al., 2008), inhibits cell replication and DNA repair, which promote apoptosis (Yamashita and Kawanishi, 2000) and inhibits multidrug-resistant proteins (Rao et al., 2012) among other effects. Quercetin is an antioxidant, protecting against lipid peroxidation, with effective antiulcer activity against ethanol-induced ulcerogenesis (Coskun et al., 2004); it also increases the level of beta-carotene and decreases the level of retinol (Bando et al., 2010). All these properties indicate the potential applications of this remarkable plant.

3.3.2 Tannins

Tannins are naturally occurring water-soluble polyphenols found in plants. Tannins have anti-microbial properties, as well as anti-carcinogenic and anti-mutagenic properties, potentially because of their antioxidant capabilities (Chung et al., 1998). Several researchers have described the presence of tannins in *T. procumbens* (Kumar et al., 2012, Edeoga et al., 2005). Acetone-water or Chloroform-water showed the presence of tannins in leaf extracts of *T. procumbens* (Table 3, Sawant and Godghate 2013). Tannins are present in the pedicle and buds of *T. procumbens* (Ikewuchi, 2012).

3.3.3 Carotenoids

Carotenoids are fat-soluble pigments found in the leaves (Ikewuchi et al., 2009) that have three main functions in a plant: light-harvesting, protection from photooxidative damage, and pigmentation to attract insects. Carotenoids have been postulated to prevent damage to DNA by oxidative stress (Wagener et al., 2012). Many types of these secondary metabolites have been isolated from *T. procumbens* including beta-carotene, which can be converted to vitamin A (Ikewuchi et al., 2009), which is important for maintenance of epithelial tissues. Vitamin A deficiency can result in impairment of immunity and hematopoiesis, night blindness, and Xerophthalmia (Sommer, 1995). Carotenoids such as beta-carotene and lutein have shown activity in the reduction of UV-induced erythema (Heinrich et al., 2003). The photoprotective properties have also been linked with the antioxidant properties of carotenoids (Wagener et al., 2012).

3.3.4 Alkaloids

Alkaloids are defined as any class of nitrogenous organic compounds of plant origin that have pronounced physiological effects on humans. The presence of some alkaloids has also been reported in *T. procumbens* (Kumar et al., 2012). In a phytochemical screening analysis, using aqueous extraction of the leaves, thirty-nine alkaloids were present, mainly Akuamidine (73.91%) and Voacangine (22.33%) (Ikewuchi, 2012). Besides alkaloids, the extract contained sterols and tannins. Alkaloids of the pedicle and buds of *T. procumbens* showed antimicrobial activity against *Proteus mirabili*s and *Candida albicans;* alkaloids from buds showed activity against *E. coli* and *Trichophyton mentagrophyte*s. The total amount of alkaloids in the pedicle was 32.25mg/gdw in the pedicles and 92.66mg/gdw in the buds (Jindal and Kumar, 2012). The presence of these alkaloids point once more to the great potential of this plant.

3.3.5 Saponins

Saponins are steroidal glycosides that contain pharmacological and medicinal properties (Atelle et al., 1999) and have been detected in *T. procumbens* (Edeoga et al., 2005), specifically a steroidal saponin and pB-Sitosterol-3-O-β-D-xylopyranoside in the flowers of the species (Saxena and Albert 2005). Another study determined that saponins from an ethanolic extract of *T. procumbens* could potentially contain antidiabetic properties by inhibiting the sodium glucose co-transporter-1 (S-GLUT-1) in the intestines of male Wistar albino rats (Petchi et al., 2013).

4. Pharmacological Properties

The great variety of secondary metabolites in *Tridax*, show the potential pharmacological properties of this species (Table 4), however, we have yet to see the use in allopathic medicine. These compounds have been used for their properties in anemia prevention, liver protection, immuno-enhancement, antioxidant, anticancer, antibacterial, antifungal, antiparasitic, antiplasmodial, and antiviral activities. This species could provide a bridge between traditional medicine and western medicine due to its pharmacological potential. More isolation and characterization of active components is needed. There is no research indicating whether there are changes in activity during the preparation and isolation of the pharmacological compounds.

Validation in table 4 is still required; for example, Ali et al. (2001) describes the isolation of flavonoids from aerial parts, but there is no correlation of the flavonoid procumbenetin to the antifungal activity. In other cases (Policegoudra et al., 2014), 26 compounds with putative antifungal activity were described but there is no reference to the phytochemicals responsible for the activity. In the work of Taddei and Romero (2002) there is no antimicrobial activity against *Candida albicans* contradicting the work done by Policegoudra and collaborators. It is possible that this is due to the different procedures used or to the type of bacterial strains used. Taddei and Romero used a three-extraction method for 7 days using dichloromethane (1:1; 3x 1000 ml) and further extraction of the aqueous layer with n-hexane followed by ethyl acetate, these authors also used paper disks for analysis and did not indicate the source of bacterial strains. Policegoudra fractionated the methanol extract with dichloromethane, used known bacterial strains and used the agar-well diffusion method. This indicates that additional work needs to be done to resolve the issue.

Table 4. Pharmacological properties of *Tridax procumbens*

Pharmacological Properties	Effect	Phytochemical	Extraction	Citation
Antimicrobial Activity	*Bacillus Faecalis, B. subtilis, E. coli, Pseudomonas aeruginosa,* Antibacterial and fungal infections	Alpha and Beta Pinenes, Alkaloids	petroleum, ether and ethanolic extracts from leaves, essences	Jhample et al., 2015 Manjamalai et al., 2012b; Pai et al., 2011
Antifungal Activity	dermatophytes, *Microsporum fulvum, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Candida albicans, and Trichosporon beigelii*	Flavonoids, Monoterpenes, and Alkaloids	Aerial parts- pedicle and buds	Ali et al., 2001; Petchi et al., 2013; Policegoudra et al., 2014
Antibacterial Activity	*Bacillus cereus, Mycobacterium smegmatis, E. Coli, Staphylococcus aureus, Klebsiella sp., Salmonella group C, Salmonella paratyphi, and Streptococcus pneumoniae*	Alpha and Beta Pinenes	N-hexane extracts, ethyl acetate extract, essential oil extract, chloroform extract	Taddei and Rosas-Romero, 2000, Manjamalai et al., 2012b; Dhanabalan et al., 2008
Antiparasitic activity	Malaria, dysentery, colic, and vaginitis, anti-Leishmaniasis activity	(3,S)-16,17-Didehydr ofalcarinol an oxylipin.	bioassay guided fractionation with a methanol extract	Martín-Quintal et al., 2009
Antioxidant Activity	Antioxidant, anti-inflammatory, anti-cancer.	High phenol content , Flavonoids (in water phase), Carotenoids (in lipid phase), Alkaloids	Ethyl acetate and n-Butanol fractions obtained from methanolic extracts, essential oils	Saxena et al., 2013; Habila et al., 2010; Han et al., 2012; Manjamalai and Berlin Grace, 2004, Jachak et al., 2017.
Anticancer Activity	Potent cytotoxic activity against malignant tumor cells.	5(alpha)- cholestane, monoterpenes (alpha and beta pinenes)	Crude flower aqueous and acetone extracts, essential oil extract	Vishnu et al., 2011; Manjamalai et al., 2012a; Policegoudra et al., 2014
Hepatoprotective Activity	Reduction of oxidative stress, lowered levels of serum Aspartate aminotransferase, serum Alanine aminotransferase, serum Alkaline phosphatase, and serum bilirubin in rats	Alkaloids, Flavonoids	Flowers, leaves, and aerial parts. chloroform insoluble fraction of an ethanol extract, petroleum ether, methanol, and chloroform water extracts, Lipopolysaccharide chloroform-insoluble fraction, aqueous extracts	Ravikumar et al., 2005a; Ravikumar et al., 2005b; Patel et al., 2014; Nwange, 2008.
Immunoenhancement Activity	Activation of the immune system with an increase of percent in neutrophils in rats	Sequesterpene and triterpenoids	No Data Found	Tiwari et al., 2004
Antidiabetic Properties	antidiabetic activity that is comparable to the drug Glibenclamide in rats.	Saponins	Ethanolic extract of whole plants, pet ether, methanol, and chloroform extracts	Sonawane et al., 2014; Petchi et al., 2013
Antihypertensive Activity	Antihypertensive activity comparable to the drug captopril in rats	Flavonoids and potentially alkaloids	ethyl acetate and dichloromethane fractions from the aerial parts of the plant	Adjagba et al., 2015

4.1 Antimicrobial Activity

Antimicrobial screenings have been done, but additional studies are needed to corroborate some of the results. Various species of bacteria and fungi have shown sensitivity to the antimicrobial properties of *T. procumbens*. More recently, callus of stem and leaf has shown to be useful for the synthesis of silver nanoparticles that showed some antimicrobial activity against *E. coli, V. cholerae, A. niger,* and *A flavus* (Bhati-Kushwaha and Malik, 2014). However, this activity was lower than the activity shown by silver nitrate so these results are not conclusive.

Petroleum, ether and ethanolic extracts of leaves of *T. procumbens* showed antibacterial activity against *Bacillus faecalis*. This activity was reported to be probably due to the presence of alkaloids. The chloroform extracts showed antibacterial activity against *B. faecalis*, *B. subtilis*, *E. coli,* and *Pseudomonas aeruginosa* (Christudas et al., 2012) but the experiments need better controls and descriptions of the procedures. Essences from *T. procumbens* show the presence of alpha and beta pinenes, used in small quantities can help in treating bacterial and fungal infections (Manjamalai et al., 2012b). There are some contradictory results about the antimicrobial activity of this species (e.g. Policegoudra et al., 2014; Taddei and Romero, 2002). Some studies did not include significant biological activity compared to the antibiotic control (e.g. Jhample et al., 2015) but there is evidence for the potential of this species as anti-microbial so more studies need to be done in this area

4.1.1 Antifungal Activity

Antifungal activity of *T. procumbens* has been investigated. Different extraction methods have been used to find the optimum zone of inhibition from different fungal strains including *Microsporum fulvum, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Candida albicans*, and *Trichosporon beigelii*. Extracts of the aerial parts of this plant have shown activity against dermatophytes with zones of inhibition ranging from 17 to 25mm with dichloromethane (DCM) fraction resulting in the best response (Policegoudra et al., 2014). However, the authors do not describe which ones are the bioactive compounds responsible for the antifungal properties. The authors suggest that these compounds could be fatty acid derivatives and constituents but no evidence is given about this statement.

4.1.2 Antibacterial Activity

Tridax procumbens has shown to have antibacterial activity. It is one of the most common plants for treating bacterial infections in rural parts of the world (Taddei and Rosas-Romero, 2000). *Tridax* extracts have shown to be effective against a variety of bacteria. N-hexane extracts have activity against *Mycobacterium smegmatis*, E. *coli, Klebsiella* sp., S*almonella* group C, and *Salmonella paratyphi*. The ethyl acetate extract was effective against Gram-positive bacteria such as *Bacillus cereus, Mycobacterium smegmatis, Staphylococcus aureus*, and Gram-negative bacteria such as *Klebsiella* sp. (Taddei and Rosas-Romero, 2000). The essential oil extract of *T. procumbens* shows significant activity against Gram-positive bacteria: *Staphylococcus aureus* and *Streptococcus pneumoniae* (Manjamalai et al., 2012b). There are some differences in how the studies were conducted so even though there seem to be strong support for the antibacterial activity of this species, more comprehensive research needs to be done.

4.1.3 Antiparasitic Activity

Treatment of some diseases caused by protozoal infections like malaria (Appiah-Opong et al., 2011; Komlaga et al., 2015), dysentery, colic, and vaginitis have been assessed with *T. procumbens* through a bioassay guided fractionation with a methanol extract to isolate an active compound, (3,S)-16,17-Didehydrofalcarinol (an oxylipin). *Tridax* seemed to have anti-leishmanial activity when using crude extracts from the whole plant (Martín-Quintal et al., 2009). A study done in Ghana tested the antiplasmodial effect of aqueous, chloroform, ethyl acetate, and ethanolic extracts from the flowers, leaves, and stem of *T. procumbens.* There is evidence that the aqueous and ethanolic extracts from the species have anti-plasmodial properties; a study using the tetrazolium-based colorimetric assay showed that *T. procumbens* helped protect red blood cells from *P. falciparum* damage (Appiah-Opong et al., 2011). Tridax shows a great potential against a disease that kills millions of people around the world.

4.2 Antioxidant Activity

Free radicals are molecules that have an unpaired electron in an atomic orbital making them highly reactive. Some of these free radicals include reactive hydroxyl radicals (OH), superoxide anion radicals, hydrogen peroxides, reactive oxygen species (ROS), and peroxyl. The instability of these radicals can damage many biologically important molecules like DNA and macromolecules, thus leading to cell damage and homeostatic disturbance. An antioxidant or a free radical scavenger is used to reduce this activity by preventing the oxidation within a biological system. Agrawal et al. (2009) analyzed the antioxidant activity of *T. procumbens* and found significant activity (comparable to the activity of Ascorbic acid) in the ethyl acetate and n-butanol fractions obtained from methanolic extracts, when using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Saxena et al., (2013) also reported a high antioxidant activity of *Tridax* when using n-butanol and ethyl acetate fractions from methanolic extracts. Habila et al., (2010) found a 96.7% antioxidant activity at a concentration of 250 µg/mL. The authors report a high reductive potential in *Tridax* (0.89 nm) compared to the standard (0.99nm) and postulate that this strong antioxidant activity could be due to the high phenol content of the plant, making this plant a good natural source of antioxidants with potential medicinal value. *T. procumbens* is also said to reduce lipid peroxidation as well as induce enzymatic and non-enzymatic antioxidants. The hepatoprotective nature of the plant may be due to flavonoids, which have been known hold free radical scavenging properties (Ravikumar et al., 2005b). The strong anti-oxidant activity of *T. procumbens* is due to the high content of phenols, flavonoids, anthraquinone, carotenoids and vitamins A and C (Nisha, 2011). All the studies report strong support for the antioxidant properties of *Tridax*.

The essential oils of *T. procumbens* have shown antioxidant activity by reducing the levels of oxidative stress when using the DPPH assay. These essential oils seem to have higher antioxidant activity than ascorbic acid and increasing the concentration of the essential oil seemed to increase the antioxidant power. It is postulated that this characteristic of *T. procumbens* makes it a great candidate for the treatment of inflammation and cancer with

less toxic effects (Manjamalai and Grace, 2004) but these claims are not properly researched and documented. For example, *T. procumbens* has shown to reduce inflammation when applied as a leaf poultice and it has shown to be effective in the treatment of neuropathic and inflammatory pain in rodent models (Sawant et al., 2014). Extract from the leaves of the plant decreased the severity of carrageenan-induced rat paw inflammation. *T. procumbens* extract at dosages of 100mg/kg, 200mg/kg, and 400mg/kg did a better job of reducing edema than aspirin at the same dosages. The plant extract did not produce ulceration and proved to be safer than aspirin and phenylbutazone (Diwan et al., 1989). Another study done more recently showed similar results. *T. procumbens* aqueous extract from the leaves showed to reduce carrageenan-induced paw inflammation. In this study the plant extract was compared to Ibuprofen instead of aspirin (Awasthi et al., 2009), but both studies show the positive effect of *Tridax* in reducing inflammation without the potential issues that could arise from the use of Aspirin or Ibuprofen.

4.3 Anticancer Activity

Cancer is a multifactorial disease. Only until recently has the anticancer activity of *T. procumbens* been researched. Crude flower aqueous and acetone extracts were tested on prostate epithelial cancerous cells (PC3). Very weak anticancer activity was observed with the aqueous extract. The acetone extract showed an 82.28% activity against cancer cells within 24 hours of treatment (Vishnu et al., 2011). The viability was analyzed using the MTT assay. The authors don't explain the toxicity analysis so the results are inconclusive since the only extract that had effect was the acetone extract and the controls are not clearly indicated in the publication. This study also does not compare the results to standard therapeutic drugs and there is no report of the selectivity index.

Significant inhibition of tumor nodule formation in the lungs was observed when using *T. procumbens*, probably due to the inhibition of formation of new blood vessels in response to monoterpenes (alpha and beta pinenes). There was also an increase of expression with P53 and caspase; indicating that the oils of this plants could induce apoptosis. Different studies have indicated that *T. procumbens* shows promise in the treatment of cancer, but more research needs to be done in order to understand the molecular mechanisms involved in this activity (Manjamalai et al., 2012a). In addition, none of the work done on anticancer activity followed the proper protocols for research in this area so the research is inconclusive.

4.4 Hepatoprotective Activity

Many models have been used to evaluate the effect that *T. procumbens* has on reducing oxidative stress in the liver, which leads to liver injury, and the hepatoprotective activity of different extracts. The chloroform insoluble fraction of an ethanol extract is effective for alleviating liver stress caused by pharmacological agents that create the same pathologies as viral hepatitis, drug intoxication, and lipid peroxidation from a reactive oxidative species (Hemalatha, 2008). A different study showed that the chloroform insoluble extract of the ethanol extract reduced hepatotoxic activity by reducing the amounts of different enzymes in rats that had been treated with CCl_4 (Saraf and Dixit, 1991). Research done on male albino rats evaluated the use of *T. procumbens* as a treatment for liver damage caused by Paracetamol (acetaminophen). It was determined that when the ethanolic extract from *T. procumbens* was administered orally at varying dosages, it lowered the levels of serum Aspartate aminotransferase, serum Alanine aminotransferase, serum Alkaline phosphatase, and serum bilirubin, resulting in hepatoprotection (Wagh and Shinde, 2010). Petroleum ether, methanol, and chloroform water extracts from flowers showed protection against hepatotoxicity in Male Wister Albino Rats, with the methanolic extract showing the best effect (Patel et al., 2014). Aqueous extracts of leaves have shown hepatoprotective activity in rats because of the antioxidant activity of these extracts, due to the active free radical scavenging (Nwanjo, 2008). An ethanolic extract from leaves of *T. procumbens* that was fractionated with chloroform showed good hepatoprotective activity in rats that had induced hepatitis by d-Galactosamine Lipopolysaccharide. The study suggests that pretreatment with the plant extract may have caused parenchymal cell regeneration in the liver. The rats that were pretreated also restored their lipid levels to normal after being treated with d-Galactosamine Lipopolysaccharide. Rats that were treated with only the *T. procumbens* extract showed to no adverse reactions, suggesting that the plant has little to no toxicity in rats. The hepatoprotective activity appeared to be from the presence of flavonoids (Ravikumar et al., 2005a). The hepatoprotective properties of *Tridax* seem to be promising and warrants future research.

4.5 Immuno-enhancement Activity

Various bioactive compounds have aided in normalization of immune response to assuage certain diseases. An adaptogen of *Tridax procumbens* has shown to enhance the body's nonspecific resistance against pathogens. Various tests in mice evaluated the effect of *Tridax* in stimulating the immune system, including the use of Swiss

Albino Mice treated with immunomodulators present in *T. procumbens* and shown to activate the immune system. This work compared the Delayed-type hypersensitivity (DTH) in the animals fed with the extracts versus the controls to evaluate cell-mediated immunity. In addition, the neutrophil adhesion was investigated showing a dose-dependent increase in the DTH response and an increase in the percentage of neutrophils. The authors suggest that there was enough evidence for the initiation of clinical trials in immunocompromised patients (Agrawal et al., 2011). However, we think that more in-depth studies should be done before clinical trials can be initiated. Even though research has shown that *T. procumbens* does possess immunostimulators, it is unclear what constituents are immunostimulators, and what constituents are immunosuppressants; different extraction and fractionation methods need to be done and then each solution tested to determine the constituents (Tiwari et al., 2004) and their activity.

4.6 Antidiabetic Properties

Diabetes has become a worldwide epidemic; interestingly, *T. procumbens* has shown antidiabetic properties. Streptozotocin-induced Male Wistar albino diabetic rats were given ethanolic extracts from the whole plant of *T. procumbens*. The study showed that the extract had antidiabetic activity that is comparable to the drug Glibenclamide used to treat diabetes mellitus type 2. The drug works by increasing the amount of insulin produced by the pancreas (Petchi et al., 2013). This study included proper controls and two different concentrations of whole plant extract of *Tridax* (250 mg/Kg and 500 mg/Kg). ANOVA and Dennett's post hoc test showed significant antidiabetic activity compared to the controls. The extracts also showed a positive effect against hyperlipidaemia associated with diabetes mellitus.

Another study showed that Alloxan-induced diabetic male albino rats responded better to methanolic extracts of *T. procumbens* than to the common drug Glibenclamide. The plant extracts were given to rats in 250 or 500 mg/kg doses, while the Glibenclamide was given at a 10 mg/kg dose. The results showed that either dosage of the plant extract lowered the blood glucose levels in the rats by 10.96%-13.74% better than the conventional drug after 6 hours of treatment. The plants extracts also showed an improvement in the fasting blood glucose levels of the Alloxan-induced diabetic rats. There was also no evidence of adverse side effects of *Tridax*'s methanolic extracts on the diabetically-induced animals. The effects of the plants on the rat's body weight was also studied (Pareek et al., 2009).

In a study done by Bhagwat et al. (2008), oral administration of aqueous and alcoholic extracts from the leaves of *T. procumbens* significantly decreased blood sugar levels in Alloxan-induced Wistar diabetic rats. The rats were given the extract for seven consecutive days at a dosage of 200mg/kg. The authors do not specify the mechanism of action of the *Tridax* extracts but this study corroborates other studies on the antidiabetic properties of this species.

T. procumbens slowed the rate of both alpha amylase and alpha glucosidase enzymes with ether, methanol, and chloroform extracts showing a significant reduction, enough to resemble common drugs used to slow the enzymes in diabetes treatment (Sonawane et al., 2014). Alpha-amylase and the Alpha-glucosidase enzymes are responsible for the breakdown of carbohydrate molecules, by slowing their breakdown rate, allowing the body to digest these carbohydrates in lower doses and therefore slowing the need for insulin, which is the main chemical affected in diabetes mellitus (Sonawane et al., 2014). All these studies demonstrate the great pharmacological potential of *Tridax* against diabetes and the importance of further research and clinical studies that could evaluate the effect in humans.

4.7 Antihypertensive Activity

For adults over 20, hypertension, or high blood pressure, is any measurement where the systolic number is above 140 mmHg, and the diastolic reading is above 90 mmHg. The CDC also characterized people who were taking medications to lower their pressure as individuals with hypertension. From 2009-2012, 30% of Americans, over the age of 20, had high blood pressure (National Center for Health Statistics). In Benin and other countries, *Tridax procumbens* has been traditionally used for the treatment of hypertension (Salami et al., 2017; Adjagba et al., 2015). Because of its traditional history, a study was done looking into its antihypertensive activity. The aerial parts of the plant were used to make cyclohexane, micellar, dichloromethane, and ethyl acetate fractions from a crude aqueous extract. Rats were treated with 20 mg/kg of N (G)-Nitro-L-Arginine-Methyl Ester (L-NAME) for seven days to induce hypertension; they were then treated with the different extracts for seven more days. The ethyl acetate and dichloromethane fractions were most effective in lowering the mean arterial pressure of the rats. The data was comparable to the effect that the common drug captopril had on the rats. Both the ethyl acetate and dichloromethane fractions contained alkaloids and flavonoids, potentially showing that those phytochemicals are responsible for the lowering of the blood pressure. There are several ideas for what the

mechanism of action is; one thought is that flavonoids can be responsible for vasorelaxation, which helps lower blood pressure. It is also said that flavonoids may have a diuretic effect that may also explain part of the plants antihypertensive activity (Adjagba et al., 2015).

5. Discussion

This review shows the importance and need to continuously research plants known to be used in traditional medicinal that could lead to the discovery and creation of new conventional medicines. *Tridax procumbens* has a long history of traditional use but isolation and evaluation of each phytochemical has not been properly related to its pharmacological properties and could show difficulty in reproducibility after isolation and evaluation. Different extracts have been used for isolation of metabolites and for treating different ailments. Based on the reviewed material many extraction studies analyzed did not do confirmatory work and some studies contradicted others. It appears that many of the extraction methods show some positive effect in a variety of disorders. Data indicates a positive effect of Tridax as an anti-diabetic when compared to conventional medicine. At the time of the writing of this review, there was no research indicating the concentration of specific phytochemicals in different plant organs, thus, determining dosage based on traditional uses is not possible. Future research needs to focus on the connection between specific phytochemical and their effects on various ailments. Others areas that have yet to be studied in depth include, but are not limited to yield of extraction, concentration and physiological activity of these phytochemicals. Discoveries in these areas will provide important information that could be used by the health community for preventative medicine and/or the discovery of new drugs. *T. procumbens* still has many important properties that remain to be discovered.

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Analysis of the *rrl3* Mutants Reveals the Importance of Arginine Biosynthesis in the Maintenance of Root Apical Meristem in Rice

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Abstract

We characterized *reduced root length3(rrl3)* mutantsof rice that exhibit a short-root phenotype under conditions producing mechanical impediments to growth, such as aerated water culture medium. The mutants were not able to maintain the quiescent center (QC) identity and produced disorganized root apical meristem (RAM) under aeration because of impaired cell division. A map-based cloning approach showed that *RRL3* encodes carbamoyl phosphate synthetase (CPS) which is thought to be required for the conversion of ornithine into citrulline during arginine biosynthesis. The *RRL3* gene is expressed highly at the root tip area that includes the root cap and division zone. The *RRL3* gene expression level was greatly affected by aeration treatment, indicating that the spatiotemporal expression of the *RRL3* gene with respect to the aeration is important for the maintenance of RAM. Furthermore, the application of citrulline and arginine could rescue the root phenotype, which implied that arginine biosynthesis was impaired in the *rrl3-1* mutant. These results suggest that the *RRL3* regulated arginine biosynthesis is important for the maintenance of RAM organization in the presence of mechanical impediments.

Keywords: root apical meristem, carbamoyl phosphate synthetase, arginine biosynthesis, mechanical impediments, rice, mutant

1. Introduction

Roots are important to plants for a wide variety of processes, including nutrient and water uptake, anchoring and mechanical support, and storage functions, and they act as the major interface between the plant and various biotic and abiotic factors in the soil environment (Gewin, 2010; Herder, Van Isterdael, Beekman, &Smet, 2010; Smith &Smet, 2012). The root length is one of the most important and thus one of the most frequently measured parameters related to the plant ability of acquiring soil resources (Kashiwagi, Iwama, & Hasegawa, 2000; Teo, Beyrouty, Norman, &Gbur, 1995). Root length is determined by the number of proliferating cells and their final size (Beemster& Baskin, 1998). In the root apical meristem (RAM), cells undergo repeated rounds of cell division in the division zone, subsequently exiting the division zone to enter the elongation zone where they undergo significant gain in size followed by differentiation. Root meristem contains a distinct central region of mitotically inactive cells termed as the quiescent center (QC) (Clowes, 1956). Stem cells within the meristem are maintained in a specialized cell environment, called the stem cell niche (Scheres, 2007), and the identities of those stem cells are regulated by the signals that originate from the QC (van den Berg, Willemsen, Hendriks, Weisbeek, &Scheres, 1997). Mutations that disrupt the functions of the stem cells or those of the QC often lead

to an impaired root phenotype in Arabidopsis (Benfey et al., 1993; Di Laurenzio et al., 1996). To date, many studies on rice root mutants have been published that have enhanced our understanding about the emergence and development of rice roots under non-stress conditions (Liang &Ichii, 1996; Ichii& Ishikawa, 1997; Inukai, Miwa, Nagato, Kitano, & Yamauchi, 2001; Inukai, Miwa, Nagato, Kitano, & Yamauchi, 2003; Scarpella, Rueb, & Meijer, 2003; Yao, Taketa, &Ichii, 2003; Debi et al., 2003; Yao, Mushika, Taketa, &Ichii, 2004; Jiang et al., 2005; Inukai et al., 2005, Kitomi, Ogawa, Kitano, &Inukai, 2008; Kitomiet al., 2011; Kitomi, Inahashi, Takehisa, Sato, &Inukai, 2012; Inukai et al., 2012). Especially, the *WUS*-type homeodomain transcription factor*QHB*, an ortholog of *WOX5*, is suggested to have a role in maintenance of the RAM in rice (Kamiya et al., 2003). Besides, the analysis of the *Osiaa23* mutant reveals the importance of *OsIAA23*-derived auxin signaling for the postembryonic maintenance of QC in rice (Jun et al., 2011). However, the genetic information about RAM maintenance in rice is still limited (Li et al., 2006; Suzaki, Yoshida, & Hirano, 2008).

The structure of the root system and its dynamics are of particular importance in crop growth under stressful conditions. For example, mechanical stress, which shifts its strength as the soil water content decrease, is often a major limitation to root elongation in aerable soils and is important to consider in breeding programmes for drought-resistant crops (Yamauchi, Pardales, &Kono, 1996; Azhiri-Sigari, Yamauch, Kamoshita, & Wade, 2000). Therefore, it is important to understand the genetic regulation of such a response to stimuli. In a previous study, we reported that the *rrl3* mutant showed a short-root phenotype under conditions producing mechanical impediments to growth, such as aerated water culture medium (Inukai et al., 2003). Under mechanical stress condition, the cell flux in the growing region of *rrl3-1* mutant reduced significantly while the mature cell length is not different from the wild type (Inukai et al., 2003).

In this study, we report the functional mode of the novel gene *RRL3,* which encodes a member of carbamoyl phosphate synthetase (CPS), possessing a large subunit ATP-binding domain. CPS is thought to catalyze the conversion of glutamine and bicarbonate into carbamoyl phosphate and glutamate, and the resulting carbamoyl phosphate is utilized in the synthesis of arginine and pyrimidine nucleotides (Holden, Thooden, &Raushel, 1999). Arginine treatment could complement the short-root phenotype, which suggests that an impaired arginine biosynthesis occurs in the *rrl3* mutant. Based on the mutant phenotype and the expression pattern of the *RRL3* gene, we concluded that spatiotemporal expression of this gene is important in arginine biosynthesis for RAM maintenance under conditions that present mechanical obstructions to the growth of the rice plant.

2. Materials and Method

2.1Plant Growth Conditions

Seedlings of the wild types (Blue Rose and Taichung 65), *rrl3-1*, and HK8117 rice mutants were grown in nutrient-free water in a growth chamber at 29°C exposed to continuous light, with or without aeration. For the allelism test, F_1 plants derived from the crosses between *rrl3-1* and HK8117 mutants were grown under identical conditions with aeration. For the citrulline, ornithine, and arginine treatment experiments, Blue Rose and *rrl3-1* plants were grown under identical conditions supplemented with 0.0, 0.05, 0.1, and 0.5 mMcitrulline, ornithine, or arginine.

2.2 Histological Analysis

BrdU (5-bromo-2′-deoxyuridine) staining was performed according to the procedure described by Ogawa, Kitamichi, Toyofuku, & Kawashima (2006). Briefly, 4-day-old plants were grown in a hydroponic system without aeration. Then, some plants were transferred to another water culture system containing 10 μM bromodeoxyuridine (BrdU) and 1 μM 5-fluorodeoxyuridine; after 7 hr, approximately 5-mm-long segments of seminal root tip were sampled for 0 days. Then, the hydroponically grown plants were aerated for 2 days and 4 days, transferred to the BrdU and 5-fluorodeoxyuridine solution, treated for 7 hr, and sampled at each time interval. Immediately after sampling, the samples were fixed with 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4), serially dehydrated with graded ethanol, and embedded in Technovit 7100 (Okenshoji Co. Ltd.). We obtained 2-μm-thick longitudinal sections by using a microtome, and the sections were dried at 37°C. The sections were then rehydrated with 10 mM phosphate buffer, stained with Meyer Heamatoxylin (Sakura Finetek Japan Co., Ltd.) for 1 hr, washed with water for 5 min, dried at 37°C, and mounted with Eukitt. Finally, the stained samples were observed under a light microscope (Olympus) and digitally imaged.

To visualize the nuclei in the RAM cells, root tip samples were stained using 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at a concentration of 0.5 μg/mL in 0.1% (v/v) Triton X-1000 for 10 min, and then washed twice with water. DAPI-stained nuclei were observed under a stereo microscope and digitally imaged.

2.3 Map-based Cloning and Expression Analysis

To map the *RRL3* gene, linkage analysis was performed using F_2 plants derived from crosses between the *rrl3-1* mutant (japonica variety) and Kasalath (indica variety). A BLAST search was performed in the rice DNA database in the Rice Genome Research Program (rapdb.dna.affrc.go.jp) and TIGR database (htt://www.tigr.org).

For RNA extraction, the root tips were sampled zone wise; for instance, zone 1 (0–1 mm), zone 2 (1–2 mm), zone 3 (2–3 mm), and zone 4 (5 mm) from the root tip of the plants grown under non-aerated conditions at 4 days after germination (DAG). For analyzing the expression pattern in relation to the aeration, sampling was performed on 5-mm root tips before starting the aeration at 1 hr, 3 hr, 6 hr, 9 hr, 12 hr, and 24 hr at 4 DAG. TRIzol reagent (Invitrogen) was used for RNA extraction, and the extracted RNA was purified using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time) (TaKaRa) and StepOnePlus Real-Time PCR (Applied Biosystems). The expression levels of the *RRL3* gene were normalized against those of an internal control, *eEF-1*α.

3. Results

3.1Inheritance of Mutant Phenotype and Allelism Tests

We have shown previously that a recessive mutant, *reduced root length3-1* (*rrl3-1*), of the rice plant has short seminal, crown, and lateral roots; in that study, we had focused on the effects of mechanical stimulus on cell multiplication in the root meristematic zone (Inukai et al., 2003). We screened another mutant line, HK8117, which exhibited a similar phenotype of the *rrl3-1* mutant. Segregation ratio of the HK8117 phenotype in the M_3 progeny derived from an M_2 heterozygous plant fit the 3 (wild type):1 (mutant) ratio (Table 1), indicating that the mutant phenotype is also controlled by a single recessive gene. We crossed the 2 mutants, *rrl3-1* and HK 8117, with each other. All F_1 progenies from the crosses of *rrl3-1* × HK8117 mutant and HK8117 × *rrl3-1* had the mutant phenotype (Table 1). These results indicate that the mutant gene of HK8117 was an allele of *rrl3-1*, and therefore, we named the mutant gene as *rrl3-2*.

Table 1. Segregation of short root phenotype in M_3 progenies derived from selfed M_2 heterozygous plants for the HK8117 and in F_1 progenies of the crosses between *rrl3-1* and HK8117

Phenotype in M_3 Progenies		χ^2 (3: 1)	P.
Normal	Short root		
126	44	0.07	0.79
Combination		Phenotype in F_1 progenies	
(♀ × ♂)		Normal	Short root
rrl3-1 × HK8117		0	15
HK8117 × *rrl3-1*		0	12

3.2 Characterization of the rrl3 Mutants

The rrl3-1 and rrl3-2 mutants showed dimorphic characteristics for root elongation under environmental stimuli. In the water culture without aeration, the seminal root lengths of the rrl3-1 and rrl3-2 mutants were about 80% and 90% of those of the wild-type, Blue Rose and Taichung 65, respectively (Fig. 1a, b). In the aerated water culture, wild-type Blue Rose and Taichung 65 seminal root length did not vary, but the rrl3-1 and rrl3-2 mutants showed significantly shorter root lengths (Fig. 1b).

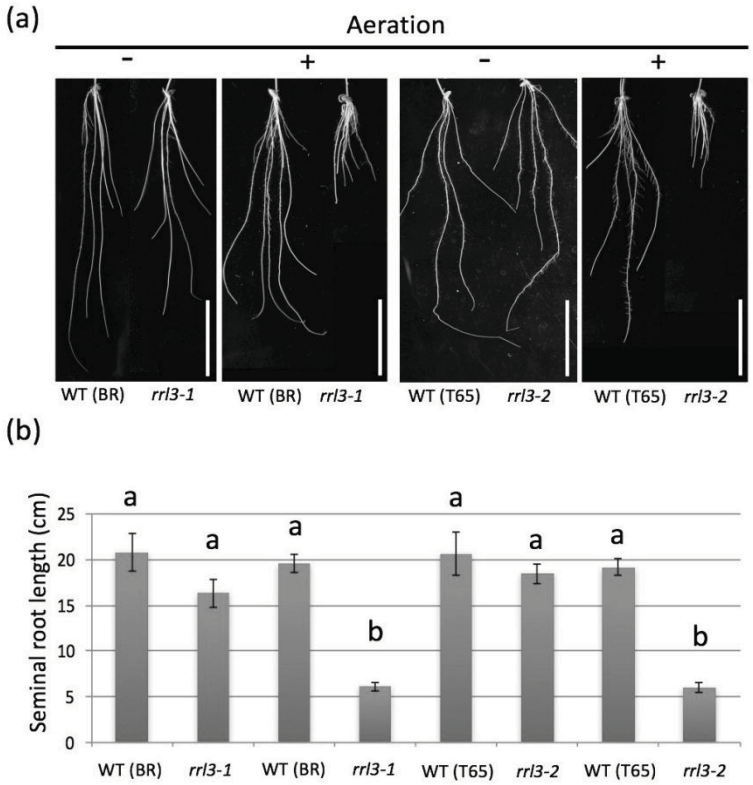

Figure 1. Phenotype of the *rrl3* mutants

Note: (a): Root phenotype of wild type, Blue rose (BR), and *rrl3-1* mutant (left group); wild-type, Taichung65 (T65), and *rrl3-2* mutant (right group); without aeration (−) and with aeration (+) at 10 days after germination (DAG). Scale bar = 5 cm. (b): Seminal root lengths corresponding to those in (a). Different letters indicate significant differences among the genotypes ($P < 0.05$) by Tukey's test.

3.3 The Short-Root Phenotype of rrl3 Mutants was a Result of the Defect in the Root Apical Meristem Maintenance

To elucidate the mechanisms by which the *rrl3* mutations affect the root elongation process, we checked the RAM phenotype by using DAPI and BrdU-labeling with respect to aeration. Before aeration, the RAM of the wild type and the *rrl3-1* mutant did not differ (Fig. 2a, h). The wild-type QC rarely divides (Fig. 2c-e). In contrast, the *rrl3-1* mutant showed the QC divided periclinally at 2 days after aeration (Fig 2j-l). With the progress of aeration, the *rrl3-1* mutant developed the meristem in which the cell size was already larger than that of the wild type (Fig. 2f, m).

We studied the cell division activity in the RAM of seminal roots of the *rrl3-1* mutant as detected by BrdU staining. The BrdU-labeled cells in the RAM of wild type and *rrl3-1* did not show any difference without aeration (Fig. 2b, i). On the other hand, 4 days after aeration, there were very few dividing cells in the division zone of the *rrl3-1* mutant compared to that of the wild type (Fig. 2g, n). Taken together, these results suggest that the *rrl3* mutant was not able to maintain the QC identity because of increased cell division resulting in a disorganized RAM; consequently, the RAM produced fewer cells and led to the short-root phenotype.

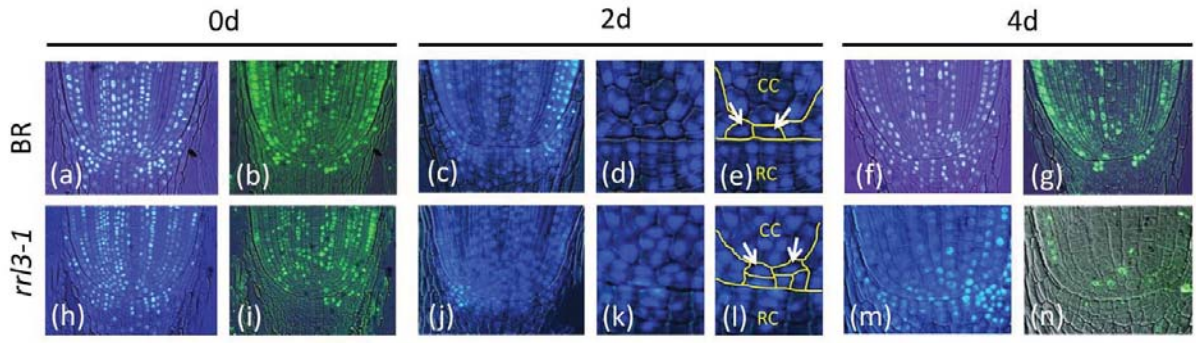

Figure 2. Changes in the RAM phenotype in the *rrl3-1* mutant grown under aeration

Note: (a) - (g): wild-type seminal roots and (h) - (n): *rrl3-1* mutant seminal roots. (a) and (h) DAPI-stained cells, (b) and (i) BrdU-stained cells in the RAM of wild type and *rrl3-1*, respectively, before aeration. (c) and (j) DAPI-stained cells in wild type and *rrl3-1*, respectively, at 2 days after aeration; (d) and (k) magnified image of the QC region of c and j, respectively. (e) and (l) showing the central cells of QC by white arrows; the *rrl3-1* mutant had dividing central cells. CC and RC indicates the central cylinder and root cap cells, respectively. (f) and (m) DAPI-stained and (g) and (n) BrdU-stained cells in the RAM of wild type and *rrl3-1* mutant, respectively, at 4 days after aeration. Scale bar = 100 μm.

3.4 Isolation of the Causative Gene in the rrl3 Mutants

A map-based cloning approach was employed to isolate the causative gene, which showed that the locus was located on the long arm of chromosome 1, approximately 90.0 cM around the molecular marker RM11602 (Fig. 3). This region includes the *RRL3* gene LOC_Os01g38970 (Os01g0570700) on the BAC clone AP003334. *RRL3* encodes a member of the CPS large subunit, CarB, which is assumed to be required for the conversion of ornithine into citrulline in the arginine biosynthesis pathway in plants. Comparison of the nucleotide sequences of the mutants *rrl3-1* and *rrl3-2* with those of the wild types showed a single-nucleotide substitution, G to A, which resulted in a single amino acid substitution, glycine (Gly) to serine (Ser) in *rrl3-1*, and C to T, which resulted in a single amino acid substitution, alanine (Ala) to valine (Val) in *rrl3-2* (Fig. 3). These results indicate that LOC_Os01g38970 is the causative gene in *rrl3* mutants.

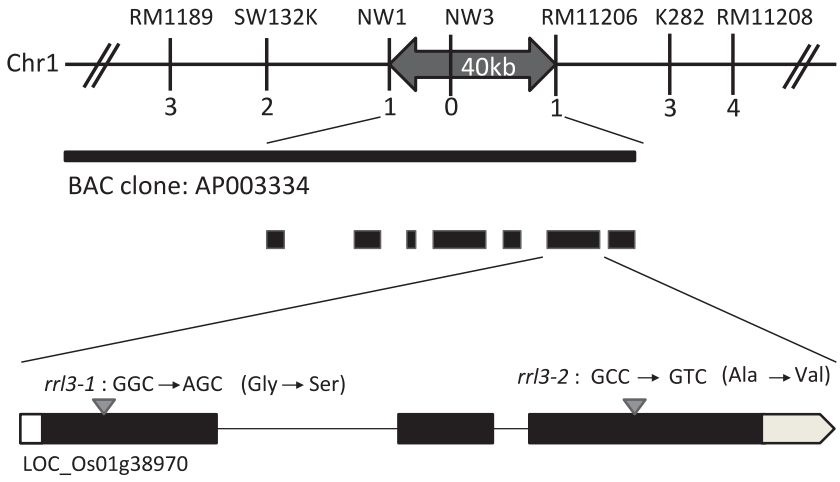

Figure 3. Map-based cloning and structure of the *RRL3* gene

Note: High-resolution linkage and physical map of the *RRL3* locus. The vertical bars represent molecular markers and the numbers of recombinant plants indicated below the linkage map. The open reading frame (ORF) of *RRL3* was 3519 bp; *rrl3-1* had a G/A substitution at 481 bp in the first exon that resulted in a single amino acid substitution of Gly to Ser, and *rrl3-2* had a C/T substitution at 2,789 bp in the third exon that resulted in a single amino acid substitution of Ala to Val. The black boxes and horizontal lines represent the exons and introns, respectively; the arrowheads indicate the mutation sites.

3.5 Expression Analysis of the RRL3 Gene

First, we checked the expression levels of *RRL3* in the 5-mm root tip segments and found that the gene is expressed in the root tip region (Fig. 4a). Then, we checked the expression pattern of the *RRL3* gene according to the different zones from the root tip of the plants grown under non-aerated conditions. Zone-wise expression patterns (Fig. 4a) revealed that the *RRL3* expression was higher in zone-1 (1 mm from the tip), which includes the root cap and division zone, than the other regions such as zone-2 (1–2 mm), which is the early elongation zone, and zone-3 (2–3 mm) i.e., the late elongation zone.

The *rrl3-1* and *rrl3-2* mutations are influenced by the environmental stimuli that are dependent on aeration, that is why we employed a time-course-based expression analysis of the *RRL3* gene in relation to the aeration. The result revealed that after starting aeration at 1 hr, the *RRL3* expression level declined, followed by a gradual increase at 3 hr; it reached the highest peak at 6 hr after aeration. After reaching the peak, the expression levels gradually decreased and returned to the normal, non-aerated levels after 2 days (Fig. 4b).

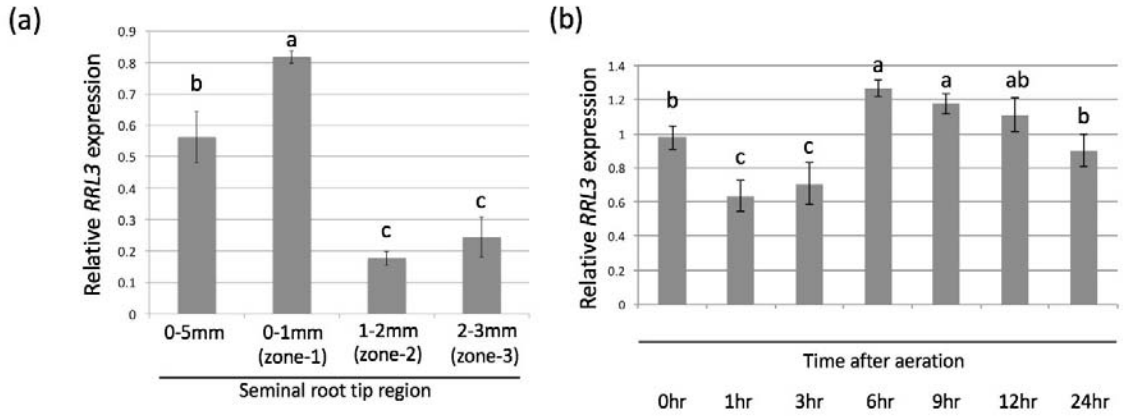

Figure 4. Expression patterns of *RRL3* gene in the wild type

Note: (a): Zone-wise expression pattern of the *RRL3,* the zone-1 (1 mm from the tip) includes the root cap and division zone; zone-2 (1–2 mm) includes the early elongation zone; and zone-3 (2–3 mm) includes the late elongation zone. (b): Time-course of the *RRL3* gene expression; 0 hr indicates before aeration; and 1 hr, 3 hr, 6 hr, 9 hr, 12 hr, and 24 hr after aeration. Different letters indicate significant differences among the genotypes ($P < 0.05$) by Tukey's test.

3.6 Application of Exogenous Arginine Biosynthesis Intermediates and Arginine Modified the Phenotype of the rrl3-1 and rrl3-2 Mutants

CPS is thought to catalyze the conversion of glutamine and bicarbonate into carbamoyl phosphate (CP) and glutamate (Holden et al., 1999). CP participates in the biosynthesis of arginine and in the *de novo* biosynthesis of pyrimidines (Zrenner, Stitt, Sonnewald, &Boldt, 2006). Ornithine and CP are the substrates of ornithine transcarbamylase (OTC), which catalyzes the synthesis of citrulline via the arginine biosynthesis pathway (Fig. 5a) (Slocum, 2005). The mutations in the *RRL3-1* and *RRL3-2* occurred in the CPS large subunit that led to the possibility of impaired arginine biosynthesis.

To examine whether the short-root phenotype of the *rrl3-1* mutant occurs because of the changes in the activity of the CPS, we grew the *rrl3-1* mutants under hydroponic condition supplemented with intermediates of the arginine biosynthesis pathway (ornithine and citrulline) as well as arginine. When we used ornithine, the root lengths of both the *rrl3-1* mutant and the wild type gradually decreased with an increasing concentration of ornithine, indicating that too high concentration of ornitine negatively affects root growth (Fig. 5b, c). In contrast, treatments with citrulline or arginine increased the root length and could complement the *rrl3-1* mutant phenotype about the wild type level at 0.1 mM. Above this concentration, the root growth of the mutant as well as that of the wild type declined probably due to also too high concentrations of them for both root growth (Fig. 5b, c). Taken together, these results indicate that the short-root phenotype of the *rrl3-1* mutants was because of a block in the conversion of ornithine to citrulline in the arginine biosynthesis pathway, resulting in the inhibition of arginine biosynthesis.

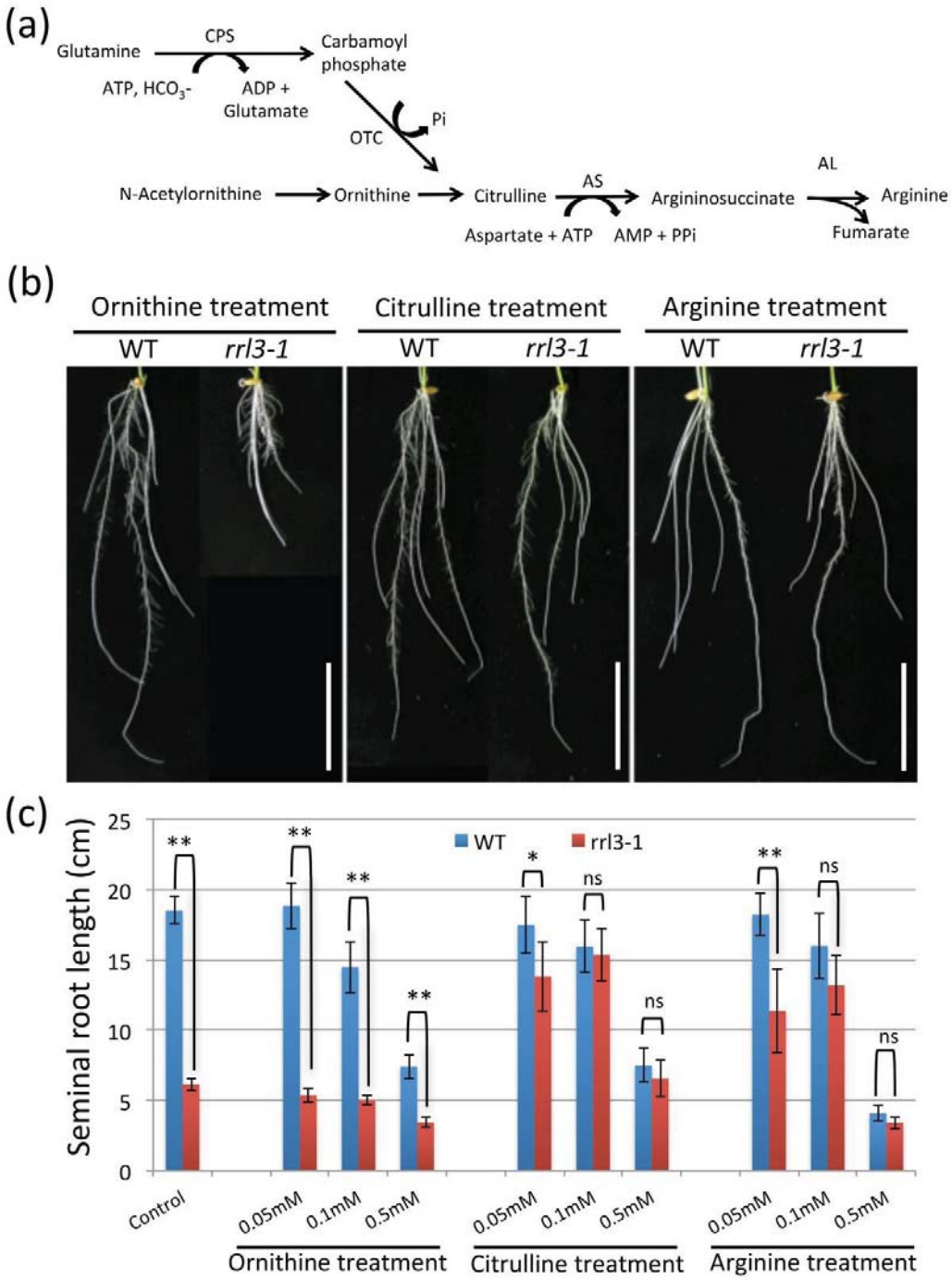

Figure 5. Effects of arginine and intermediates of arginine biosynthesis pathway in *rrl3-1* mutant

(a): Synthesis of arginine from the ornithine intermediate in plants. CPS, carbamoyl phosphate synthetase; OTC, ornithine transcarbamylase; AS, argininosuccinatesynthetase; AL, argininosuccinatelyase. (b): Root phenotype of 10-day-old plants treated with 0.1 mM ornithine (left group), citrulline (middle group), and arginine (right group); in each group, the left plant is the wild type, and the right plant is the *rrl3-1* mutant. Scale bar = 5 cm. (c): Seminal root length of the control and the ornithine-, citrulline-, and arginine-treated plants; ns, **, and * indicate not significant, significant at 1% level, and significant at 5% level, respectively.

4. Discussion

In this study, we showed that *rrl3-1* and *rrl3-2* are allelic mutants of the *RRL3* gene. *RRL3* encodes CPS large subunit, CarB, which is thought to catalyze the conversion of glutamine and bicarbonate into carbamoyl

phosphate and glutamate. The CarB protein has 2 catalytic domains (N-terminus ATP-binding, residues 104–500; C-terminus ATP-binding, residues 655–1063) that are assumed to participate in 2 different ATP-dependent reactions, which include the phosphorylation of bicarbonate (104–500) and carbamate (655–1063) (Slocum 2005). In the *RRL3-1* mutant, the amino acid substitution takes place in the N-terminal ATP-binding domain (161 residue), and in *RRL3-2*, the amino acid substitution takes place in the C-terminal ATP-binding domain (930 residue). These amino acids have been shown to be conserved in Arabidopsis CPS (Molla-Morales *et al.* 2011). This suggests that *rrl3-1* and *rrl3-2* are loss-of-function mutants of the *RRL3* gene.

In plants, a single CPS (EC 6.3.5.5) provides common carbamoyl phosphate intermediates for the synthesis of both arginine and pyrimidines, which are coordinately regulated (Slocum, 2005).As *RRL3* is present as a single copy in rice, the phenotype of mutant alleles cannot be masked by redundant homologs. The lack of known mutants in the arginine biosynthesis pathway suggests that arginine is essential for plant metabolism, and that the null alleles of a gene in this pathway are lethal at the gametophytic or embryonic levels (Molla-Morales et al., 2011). Arabidopsis *ven3* mutant resulted from the mutation in the conserved domains of CPS subunit, CarB, exhibiting differential pigmentation of veinal and interveinal tissues (Molla-Morales et al., 2011). In contrast, a T-DNA insertional mutation in the 3′-untranslated region (UTR) of the ornithine transcarbamylase (*OTC*) gene shows increased sensitivity to exogenous ornithine, but could not produce any visible phenotype (Quesada, Ponce, &Micol, 1999). Although another mutant of *Arabidopsis* with a T-DNA insertion in the promoter of *CarB* (*VEN3*) reduces the expression levels, its effect on arginine biosynthesis is negligible and it does not produce any visible phenotype (Potel et al., 2009). These 2 T-DNA insertional mutants had a mutation in the non-coding regions; therefore, these 2 mutants still retain the wild-type OTC and CarB proteins, although at reduced levels. Therefore, it is conceivable that the catalytic activities of the *rrl3-1* and *rrl3-2* genes reduced but can still maintain sufficient arginine levels for survival under normal conditions, but not in the presence of mechanical impediments to growth.

Arginine is an important constituent of proteins, and is thought to serve, under some circumstances, as a store of nitrogen for the biosynthesis of secondary products such as polyamines (Shargool, Jain, & McKay, 1988) and other amino acids during seed development (de Ruiter&Kolloffel, 1982). It has been reported that defects in overall protein biosynthesis, such as in mutants of ribosomal protein genes, caused a significant reduction in cell numbers (Horiguchi et al., 2010). In Arabidopsis *cue1* mutants, disruption of the aromatic amino acid biosynthesis causes an imbalance in the levels of other amino acids (Streatfield et al., 1999; Voll et al., 2003). Arabidopsis *ven3-2* mutant showed altered amino acids, which suggests that protein synthesis does not proceed efficiently in this mutant (Molla-Morales et al., 2011). Therefore, the mutation in the *RRL3* gene reduced the arginine levels in the RAM, causing a limited supply of other amino acids for the biosynthesis of proteins for the growing tissues in the *rrl3* mutants.

Root growth in *Arabidopsis* is impaired in the *tup5* mutant under conditions of normal light, but not in the dark. The free arginine content in the *tup5* mutant was lowered to 31% of that of the wild type under long-day conditions, but not in the dark; in addition, the free arginine content decreased and the RAM was almost completely utilized in the dark, reflecting that under normal light conditions, plants need higher levels of arginine to maintain the RAM in *Arabidopsis* (Frémont, Riefler, Stolz, &Schmülling, 2013). Consistent with these findings, we believe that rice plants may need to produce increased levels of arginine under mechanical stimuli that impede normal growth, which shows why the *rrl3* mutants could not maintain their RAM in presence of mechanical impediments. The spatiotemporal/differential expression pattern of *RRL3* with and without aeration is also consistent with the phenotype observed in the *rrl3* mutants in presence of similar environmental stimuli.

The *Osiaa23* mutant cannot maintain its QC identity because of abnormal transverse division in the QC cells. The analysis of the QC of *Osiaa23* mutants has shown that the postembryonic maintenance of the QC depends on *OsIAA23*-mediated auxin signaling (Jun et al., 2011). In contrast to *OsIAA23*, the *RRL3* gene maintains the QC through the regulation of arginine biosynthesis. Our previous research has shown that the *RRL3* gene regulates cell production in the root under a mechanically impeded condition, and does not regulate the sensitivities to ethylene, IAA, and ABA (Inukai et al., 2003). In addition, examination of the RiceXPro database reveals that the *RRL3* gene is not induced by auxin (http://ricexpro.dna.affrc.go.jp). Our data emphasize the fact that in addition to the traditional analyses of transcriptional regulation, phytohormones, and signal transduction, a complete understanding of the mechanisms regulating the meristem and initial cell function in plants must also incorporate models describing the role of essential metabolites such as arginine. Hence, we can conclude that *RRL3* is a novel gene that is involved in the production of structural units such as arginine, and ultimately, proteins that maintain the QC and RAM in the rice plant.

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Analysis of Growth Parameters for Crop Vegetables under Broad and Narrow LED Spectra and Fluorescent Light Tubes at Different PPFs

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Abstract

Several physiological and yield parameters were evaluated in lettuce plants, cv. 'Trocadero', while growing at four different photosynthetic photon flux (PPF) (70, 120, 250 and 400 ± 10 µmol m^{-2} s^{-1}), under four light spectra, white (W), red (R) and blue (B) Light-Emitting Diode (LED) lamps and cool white fluorescent tubes (FL). Yield parameters were also evaluated on spinach, turnip and radish, growing under identical light spectra but using a single PPF (340 ± 10 µmol m^{-2} s^{-1}). Lettuce development was impaired at PPFs below 250 µmol m^{-2} s^{-1} for all tested spectra. At higher PPFs (250 and 400 ± 10 µmol m^{-2} s^{-1}), for the two broad spectra tested (W LEDs and FL light), no significant differences were registered on all physiological and yield parameters evaluated. On all situations W LEDs performed, at least, as good as the FL light, indicating that actual W LEDs can efficiently replace traditional light sources, with all the inherent benefits, which include significant lower power consumption. For all species, narrow light spectra (R and B LEDs) proved not being able to provide normal plant development. Plants under R LEDs, although presenting, in some situations, a fresh weight higher than those achieved with the broad light spectra, always led to abnormal plant morphology, characterized by expanded petioles and leaf curling. B LEDs, in spite of promoting plant growth with normal morphology, frequently led to a lower number of leaves and consequently to a lower fresh weight.

Keywords: controlled environment, light intensity, light spectra, photosynthesis, plant growth, yield parameters

1. Introduction

LED light is known since 1920, however, a practical visible-light version (red), usable only as indicator lamps due to its very low power, was only developed in the early 1960s. Through the 70s, new technical developments were achieved, and lamps operated in short wavelength ranges (orange, yellow, and green) were released. The first blue LED lamp was developed in 1993 and, in 1996, a phosphor coating was applied to a blue LED to create the world's first white LED lamp (Bourget, 2008). The major advance in LED technology was the development of the first high-power (1 W or greater) lamp device, in 1999, and, in the first years of this century, LED light efficiency rapidly overcome the majority of light sources available until then, becoming as efficient as the sodium vapour lamps (Pimputkar, Speck, DenBaars & Nakamura, 2009).

As stated by Brandon et al. (2016), LED light present several advantages when compared with traditional light sources; - Lower consumption and longer lamp life; - Light intensity adjustment 0-100% (dimming); - Lower heat emission, allowing its installation near the plant canopy. The knowledge of these advantages, combined with the possibility of using specific wavelengths, have driven the interest of plant producers on the use of LED light, either as a supplement, in conditions where the natural light is not enough, or for plant production under 100% artificial light conditions.

Having the tools to build a customized light spectrum, it was the time for researchers to look for the best spectral composition which could maximize plant development and production. The need to use red light to power up photosynthesis was widely accepted for two reasons. First, due to the McCree curves (Mc Cree, 1971), which

indicate that wavelengths within the range of 600 to 700 nm are more efficiently absorbed by plant pigments, and second, because the first available LEDs were red, emitting at 660 nm, close to one of the absorption peaks of chlorophyll. Another wavelength, within the range of 400 to 500 nm, corresponding to the blue region of the visible spectrum, was also included in the early studies (Massa, Kim, Wheeler & Mitchell, 2008). Blue light is linked to several important photomorphogenic responses in plants, including stomatal control, which affects water relations and CO_2 exchange (Schwartz & Zeiger, 1984), stem elongation (Cosgrove, 1981) and phototropism (Blaauw & Blaauw-Jansen, 1970).

Studies looking for the best combination of red and blue LEDs, able to optimize plant growth in controlled environments, were done on lettuce by Okamoto, Yanagi and Kondo (1997), Yorio, Goins, Kagie, Wheeler and Sager (2001), Johkan, Shoji, Goto, Hashida, and Yoshihara (2010), Lin et al. (2013) and Borowski, Michalek, Rubinowska, Hawrylak-Nowak and Grudzinski (2015), as well as on other plant species, such as: *Lilium* (Lian, Murthy & Paek, 2002), *Chrysanthemum* sp. (Kim, Goins, Wheeler & Sager, 2004), *Withania somnifera* (Lee, Tewari, Hahn & Paek, 2007), *Doritaenopsis* (Shin, Murthy, Heo, Hahn & Peak, 2008), *Brassica rapa* subsp. *chinensis* (Li, Tang, Xu, Liu & Han, 2012), *Valerianella locusta* (Wojciechowska, Kołton, Długosz-Grochowska, Żupnik & Grzesiak, 2013). However, as stated by Mitchell (2015), growing plants under red light and then conclude that adding a little blue light makes them grow even better, is not surprising. In fact, if a little green light is added along with red and blue, plant species with some mutual leaf shading grow even better (Kim et al., 2004; Lu et al., 2012). Adding some Far-Red light (FR) plants grow taller (Brown, Schuerger & Sage, 1995; Chia & Kubota, 2010); and some photoperiodic classes of plants flower better when this light wavelength is present during night-break lighting (Deitzer, Hayes & Jabben, 1979; Kohyama, Whitman & Runkle, 2014). Some species develop physiological disorders like gall-like tumours on their leaves and shoot tips, generally due to a deficiency of ultraviolet radiation (Morrow & Tibbitts, 1988). Ultraviolet light also promotes biosynthesis of pigments and accumulation of a wide array of phytochemicals in fruits and vegetables (Li & Kubota, 2009; Samuolienė et al., 2013).

So, will we eventually need for artificial light to have a broad spectrum similar to that of the sun for maximum yield and quality? Are we gradually rediscovering the value of white light for growing plants? On such scenario, can the white LEDs be the answer?

White LEDs are mostly produced by using blue LEDs and phosphors coated caps. In the first years of this century, the efficiency of blue LEDs (3 to 4%) was low when compared with the efficiency of red LEDs (15-18%) (Massa, Emmerich, Morrow, Bourget & Mitchell, 2006). Since then, the efficiency of blue LEDs has dramatically increased, which has made possible to significantly improve white LEDs performance (Pimputkar et al., 2009). New developments also arose on white LED light production. Improvements on the efficiency of green LEDs, allowed higher performance of systems based on mixtures of red, blue and green emitting diodes. The use of purple emitting diodes, coated by a new recently-developed phosphor mixture (TRI-R white LED technology developed by Toshiba), allowed the production of white light with a spectrum closer to sunlight. The actual efficiency of LEDs is around 66% for blue, 44% for red and 33% for white based on blue phosphor-converted LED, having the last one significant margin for further improvement. In fact, an efficiency of 61%, is considered to be the upper phosphor-converted white LEDs potential.

Regardless of the way LED white-light is produced, the main limitation, its low efficiency, has been overcome. On such circumstances, is it possible for the new white LEDs to replace traditional light sources, such as fluorescent lamps, in production systems using 100% artificial light for plant growth?

In this research, two trials were conducted to evaluate the efficiency of narrow and broad band LED spectra on the development of several horticultural species. Traditional cool white fluorescent lamps were used as control.

2. Method

2.1 Plant Material and Growth Conditions

Seeds of *Lactuca sativa* L. cv. 'Trocadero', *Spinacia oleracea* L cv. 'Gigante de Inverno', *Brassica rapa* L. var. *rapa* cv. 'Bola de Neve' and *Raphanus sativus* L. cv. 'Redondo Vermelho', were used. The seeds were germinated on a plant growth chamber (15000EDTU from Aralab®, Portugal) in 84-cell plug trays filled with vermiculite (one seed/cell), under a 16 h photoperiod, a PPF of 200 ± 10 µmol m^{-2} s^{-1}, 22/20 ºC day/night temperature and 70% relative humidity.

In the lettuce trial, seedlings at the 4 to 6 leaf stage were transferred to individual pots (0, 60 L) filled with perlite and maintained under the growth conditions previously described. Seven days later, 256 plants, as homogeneous as possible, were selected and transferred to 16 light treatments, with four PPF intensities and four different light

sources (Figure 1) (see 2.3. for details). For the trial with spinach, turnip and radish, 64 seedlings per specie were selected and submitted to the same 4 light treatments used in the trial with lettuce but established under a single PPF.

PPF was measured using a quantum sensor (SKP200 from Skye Instruments, UK) and the light spectral distribution was evaluated using a spectroradiometer USB2000+ Ocean Optics FL, USA.

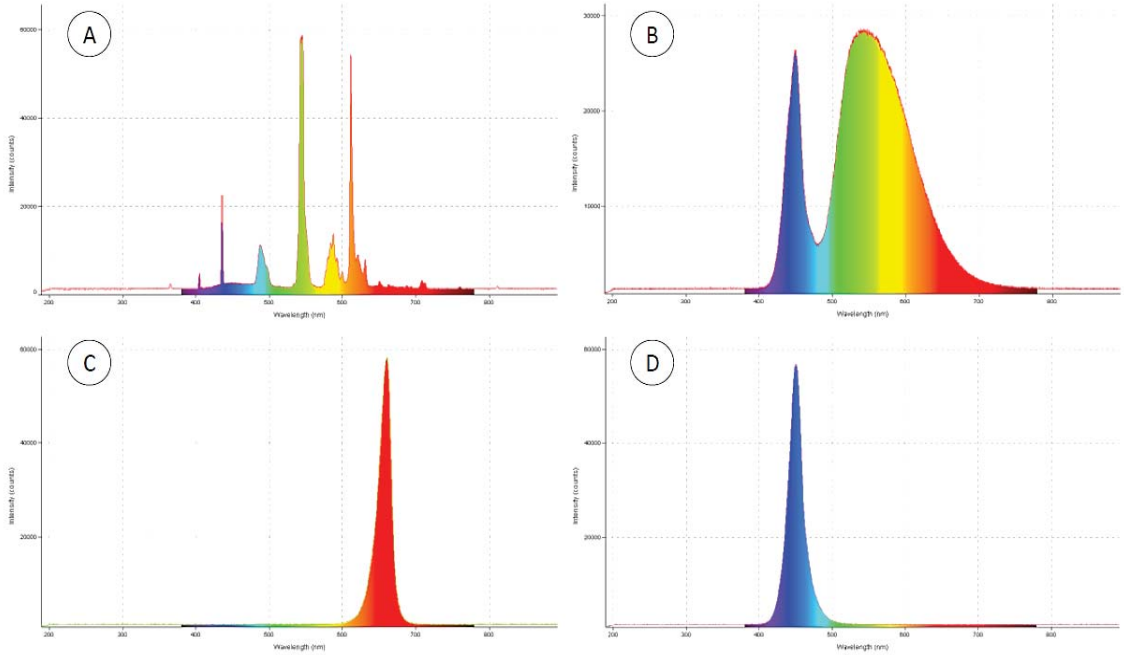

Figure 1. Spectral photon distributions of the four light treatments. (A) Fluorescent tubes; (B) White LEDs; (C) Red LEDs, (D) Blue LEDs

Plants were irrigated twice a day with a nutrient solution containing 5 mmol·L^{-1} NO_3, 1.8 mmol·L^{-1} NH_4, 0.45 mmol·L^{-1} P, 3.90 mmol·L^{-1} K, 1.2 mmol·L^{-1} Ca, 0.49 mmol·L^{-1} Mg, 0.33 mmol·L^{-1} S, 46 µmol·L^{-1} B; 7.86 µmol·L^{-1} Cu, 8.95 µmol·L^{-1} Fe, 18.3 µmol·L^{-1} Mn, 1 µmol·L^{-1} Mo, and 2 µmol·L^{-1} Zn ,1.8 mmol·L^{-1} Cl and 0.5 mmol·L^{-1} Na.

Environmental conditions provided by growth chambers Fitoclima1200PLH, from Aralab® (Portugal), were maintained with 12 h photoperiod, 22 °C/14 °C (day/night) temperature and 65/80% (day/night) relative humidity.

2.2 Plant Measurements

The physiological parameters, photosynthetic rate (A), stomatal conductance (g_s), maximum quantum efficiency of PSII photochemistry (Fv/Fm) and relative chlorophyll (Chl) content, were measured only in the trial with lettuce.

Relative chlorophyll content was measured using a chlorophyll content meter CL-01 from Hansatech Instruments (UK), while chlorophyll fluorescence analysis of maximum quantum efficiency of PSII photochemistry was measured using a chlorophyll fluorometer OS-30p+ from Opti-Sciences (USA). Leaves were allowed to dark-adapt for 30 min before measurement.

Stomatal conductance measurements were taken with porometer AP4 from Delta-T (UK) between 9:30 and 12:30 AM on two of the youngest fully expanded leaves per plant.

Gas exchange measurements were carried out with an LCi Portable Photosynthesis System from ADC BioScientific Ltd. (UK). Measurements were conducted on completely expanded mid-leaf blades.

Analysis of yield parameters included leaf number, shoot and root fresh and dry weights (fresh weight was evaluated only in the trial with lettuce), being the dry weights of leaf and root measured after oven-drying at 80ºC for 96 h.

2.3 Experimental Design and Statistical Analysis

The trial with lettuce was conducted with four PPF intensities, 70, 120, 250 and 400 ± 10 µmol m^{-2} s^{-1} and four different light spectra provided by cool white fluorescent lamps (Fl) (Philips Master PL-L 55W/840/4P), that were used as control and White (W), Red (R) or Blue (B) LEDs, provided by Philips Green Power Led Research modules. The same light spectra were used in the trial with spinach, turnip and radish, which was established using a single PPF of 340 ± 10 µmol m^{-2} s^{-1}. Sixteen plants were used on each PPF/Light Source combination. All trials were conducted at the University of Évora, Portugal.

Physiological parameters were determined only on lettuce, 35 days after seedlings were submitted to light treatments. Plants from both trials were harvested at day 40 for assessment of yield parameters.

All the collected data were submitted to ANOVA analyses followed by post-hoc evaluation using the Tukey test, being significant differences considered at $p \leq 0.05$. The STATISTICA Version 10 software (Statistica, Tulsa, USA) was used for data analysis.

3. Results

3.1 Analysis of Yield Parameters

3.1.1 Trial with Lettuce under Four Light Intensities and Four Light Spectra

From the results of yield parameters presented in table 1, it is possible to observe that PPF significantly affected all variables under evaluation, being the best values achieved when the highest PPFs (250 and 400 µmol m^{-2} s^{-1}) were used. This is particularly evident for the total dry weight and for the root fresh weigh, but it is also a general tendency for all the other variables. By increasing PPF from 250 to 400 µmol m^{-2} s^{-1}, no increase in the total dry weight was registered, and, a significant reduction on the total fresh weight was observed, especially when the narrow spectra (R and B LEDs) were used. This may be related with the saturation of photoreceptors for specific wavelengths, but can also be due to inefficient water and nutrient supply, situations that will be discussed further.

Table 1. Yield parameters for *Lactuca sativa* cv.'Trocadero'

Intensity µmol m^{-2} s^{-1} (PPF)	Light spectra	Leaf number/plant (n)	Shoot fresh weight (g/plant)	Shoot dry weight (g/plant)	Root fresh weigh (g/plant)	Root dry weight (g/plant)	Total fresh weight (g/plant)	Total dry weight (g/plant)
70	(Fl)	28 de	24.1 g	1.33 c	2.26 b	0.13 d	26.3 f	1.46 b
	(W)	29 d	29.1 fg	1.47 c	2.42 b	0.15 d	31.5 f	1.62 b
	(R)	26 de	25.2 g	1.19 c	1.50 b	0.11 d	26.7 f	1.30 b
	(B)	18 g	22.1 g	1.34 c	2.60 b	0.14 d	24.7 f	1.48 b
120	(Fl)	26 de	24.9 g	1.61 c	2.86 b	0.15 d	27.8 f	1.76 b
	(W)	31 cd	33.8 fg	2.10 c	3.48 b	0.18 d	37.2 ef	2.29 b
	(R)	30 d	38.7 efg	2.08 c	2.54 b	0.13 d	41.2 ef	2.21 b
	(B)	19 fg	22.4 g	1.58 c	3.43 b	0.18 d	25.9 f	1.76 b
250	(Fl)	36 b	81.2 bc	5.15 ab	9.78 a	0.56 c	90.3 bc	5.70 a
	(W)	36 b	95.9 b	5.81 ab	10.81 a	0.64 abc	106.7 b	6.45 a
	(R)	42 a	125.6 a	6.55 a	9.59 a	0.53 c	135.2 a	7.08 a
	(B)	26 de	75.4 bcd	6.30 ab	10.66 a	0.57 bc	86.0 bc	6.87 a
400	(Fl)	35 bc	56.8 de	5.36 ab	11.37 a	0.83 a	68.2 cd	6.20 a
	(W)	36 ab	73.5 cd	6.20 ab	10.61 a	0.79 a	84.1 bc	6.69 a
	(R)	40 ab	77.9 bc	5.86 ab	10.58 a	0.76 ab	88.5 bc	6.66 a
	(B)	23 ef	47.8 ef	4.62 b	12.23 a	0.83 a	60.1 de	5.46 a

Different letters in the same column correspond to significant differences ($p \leq 0.05$) by Tukey test.

Concerning light spectra, its effect on plant growth increased with the PPF increasing, being two variables particularly affected: the leaf number, which was negatively affected by the use of B LEDs, and the shoot fresh weight, which was positively affected by the use of R LEDs. Nevertheless, none of these negative and positive effects on plant development had impact on the shoot dry weight, where the significant differences resulted solely from PPF variation.

As it can be seen from figure 2, plants growing under R LEDs presented abnormal leaf development with elongated petioles and pronounced leaf curling. Although visible in all R LED treatments, this effect of leaf curling was reduced with the PPF increase. Also by observation of figure 2, is possible to see that when a non-limiting PPF is

used, broad spectra provided better results than narrow spectra, by producing plants with higher commercial value. Nevertheless, plants seem to have an enormous capacity to adjust photon receptors to the available wavelengths.

By comparing the two broad spectra used (FL tubes and W LEDs), for all the yield parameters under evaluation (Table 1), W LEDs always presented better results, or at least non-significantly different from those achieved with the use of fluorescent tubes.

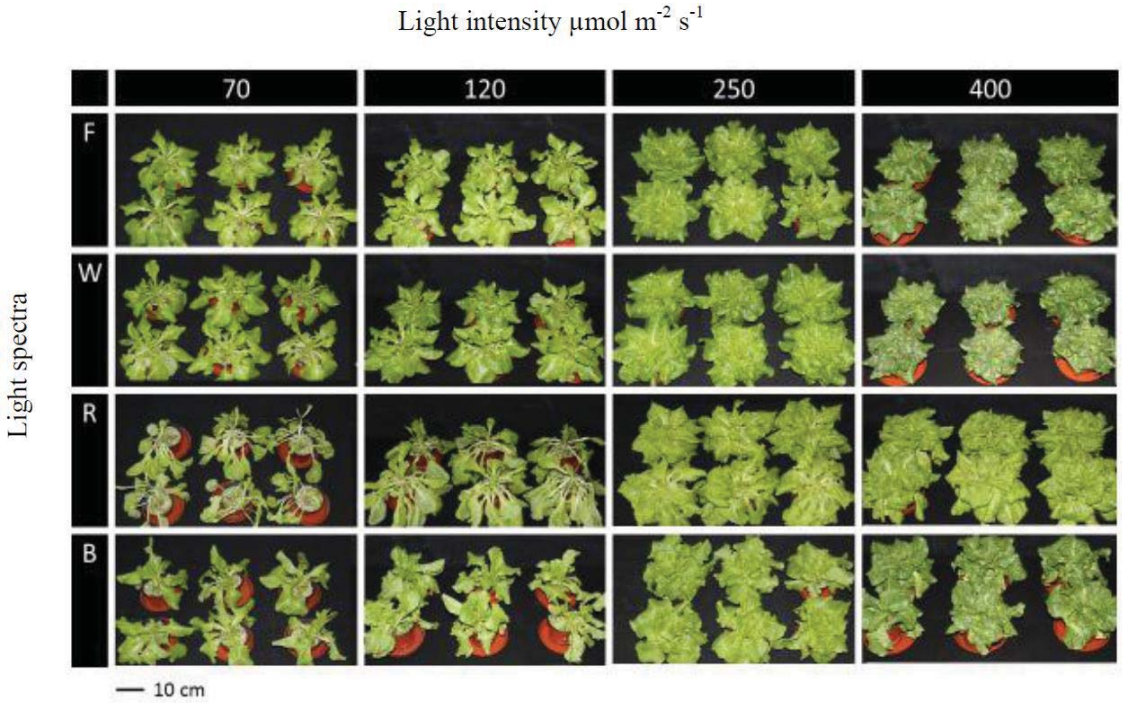

Figure 2. General aspect of plants from the lettuce trial, 40 days after having been transplanted into the four light spectra and the four PPFs tested. On the lowest PPFs, the plants present a non-compact aspect, with elongated petioles and narrow leaves, being those morphological anomalies particularly visible when the R LEDs (R70 and R120) were used. The most compact plants, with the highest commercial value, are those which evolved under a 400 μmol m^{-2} s^{-1} PPF. Nevertheless, the ones growing under a 250 μmol m^{-2} s^{-1} PPF, presented the highest shoot fresh weight

3.1.2 Trial with Spinach, Turnip and Radish, Under a Single Light Intensity and Four Light Spectra

The data collected for these three species (dry weight of shoots and roots and leaf number) is presented at table 2. Concerning dry weight, data follows the trend already observed for lettuce on higher PPFs, i.e., no significant differences being registered among spectra, neither for shoot dry weight, nor for total dry weight. For the roots, dry weight presented some differences among spectra, with R LEDs always giving the lowest values, following the same trend already observed for lettuce.

Table 2. Yield parameters for spinach, turnip and radish

Species	Light spectra	Evaluated Parameters			
		Shoot dry weight (g/plant)	Roots dry weight (g/plant)	Total dry weigh (g/plant)	Leaf number/plant (n)
Spinach	(Fl)	4.06 a	0.41 b	4.47 a	23 b
	(W)	5.28 a	0.44 ab	5.72 a	28 a
	(R)	4.44 a	0.33 b	4.77 a	30 a
	(B)	4.64 a	0.52 a	5.16 a	22 b
Turnip	(Fl)	4.05 a	0.62 a	4.67 a	10 a
	(W)	5.38 a	0.75 a	6.13 a	10 a
	(R)	5.01 a	0.55 a	5.57 a	11 a
	(B)	5.01 a	0.56 a	5.56 a	8 b
Radish	(Fl)	1.10 a	0.85 a	1.95 a	7 a
	(W)	1.23 a	0.70 ab	1.93 a	7 a
	(R)	1.32 a	0.48 b	1.80 a	8 a
	(B)	1.30 a	0.77 a	2.07 a	8 a

For each species, different letters in the same column correspond to significant differences ($p \leq 0.05$) by Tukey test.

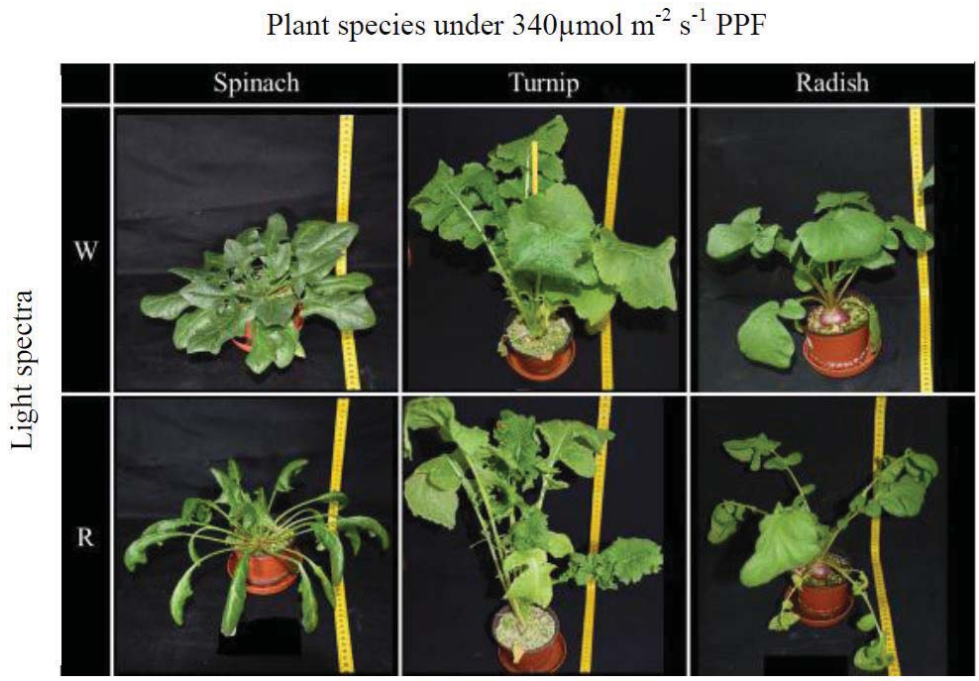

Figure 3. Aspect of spinach, turnip and radish plants growing under R and W LEDs, 40 days after transplant. As registered for the lettuce, the abnormal petiole elongation, as well as the leaf curling, can be observed

Leaf number was also lower when B LED was used. Nevertheless, this effect was not registered for radish, and, with spinach, a reduction on leaf number was also observed with fluorescent light. All plants growing under R LED light spectrum also presented abnormal leaf development, more pronounced for spinach and radish, as can be seen on figure 3.

3.2 Analysis of Physiological Parameters for the Lettuce Trial

Data related to photosynthetic rate (A), relative chlorophyll content, maximum quantum efficiency of the photochemical center of the active photosystem II (Fv/Fm) and stomatal conductance (*g*s), are presented in figure 4. It can be seen that the photosynthetic rate (Figure 4A) increased linearly with PPF increase, but, plants exposed to R LEDs, seem to have achieved maximum net photosynthesis at 250 µmol m^{-2} s^{-1}. In relation to the light spectra,

slight differences were observed on photosynthesis for 70, 120 and 250 µmol m^{-2} s^{-1} treatments, but, differences became significant, at 400 µmol m^{-2} s^{-1}. At this light intensity, W LEDs performed better than B LEDs (1,1% lower), FL (3% lower) and then R LEDs (4,2% lower).

Similar evolution patterns were observed for the chlorophyll content. However, for this parameter, on all the PPFs tested, the plants growing under R LEDs presented values of chlorophyll significantly lower than the registered for the other light spectra (Figure 4B).

Concerning the Fv/Fm values, is possible to see from figure 4C that plants plant growing under R LEDs, presented values systematically lower than 0,80. Also for FL and W LEDs, values lower than 0,8 were registered at 70 µmol m^{-2} s^{-1} PPF. The values from stomatal conductance (*gs*) are presented in figure 4D. This parameter proved to be highly dependent on the light spectra, with the highest values being always obtained from spectra enriched in blue wavelengths (B and W LEDs). The significant reduction on the *gs* values registered for the 400 µmol m^{-2} s^{-1} treatment, were probably conditioned by factors other than the light, as will be discussed below.

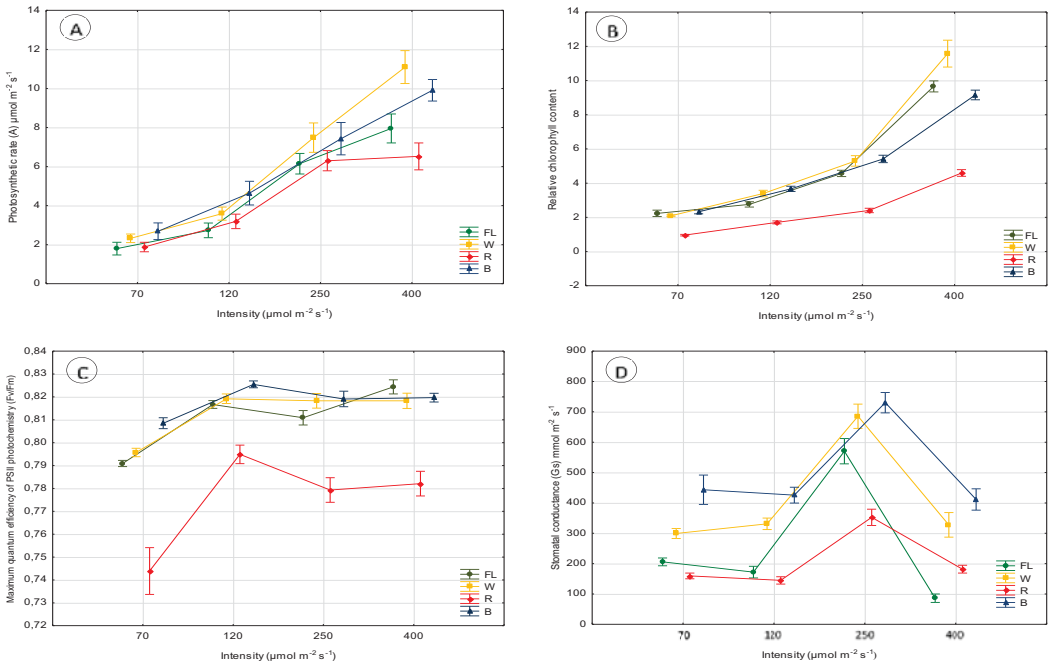

Figure 4. Behaviour of photosynthetic rate (A), relative chlorophyll content (B), maximum quantum efficiency of the photochemical center of the active photosystem II (C) and stomatal conductance (D), on lettuce plants growing under 4 light spectra and 4 light intensity regimes. Bars represent the 95% confidence intervals

4. Discussion

As stated by Snowden, Cope and Bugbee (2016), the effects of spectral quality on plant development are not well understood because much of the current understanding comes from studies where PPFs are usually less than 10% of the summer sunlight. Moreover, PPFs also change from author to author.

The light saturation point should be one of the most important parameters upon which optimal light intensity regulation is based (Fu, Li & Wu, 2012), but, conflicting results are provided by different studies. In lettuce, for instance, some showed that the saturation point is as high as 889 to 932 µmol m^{-2} s^{-1} (Knight & Mitchell 1983 a, b), while others point it near 500 to520 µmol m^{-2} s^{-1} (Glenn, Cardran & Thompson, 1984). This conflicting results on the light saturation point for a species, also do not facilitate PPF regulation in production systems.

From data now presented, it can be seen that significant differences were observed on yield and physiological parameters between the different PPFs used. Nevertheless, for PPFs lower than 250 µmol m^{-2} s^{-1}, used in lettuce, apart from the leaf number, no other differences related with the spectra were found on the yield parameters, and, in no case, was possible to obtain plants with commercial value.

The lower leaf number registered when B LEDs were used, is an issue already observed by Chen, Guo, Xue Wang and Qiao (2014) and Wang, Lu, Tong and Yang (2016), and, as confirmed by our trials, seems to be more related with the light spectra than with the PPF levels. As stated by Dougher and Bugbee (2004), blue light induces inhibition of cell expansion and division, which in turn results in fewer internodes and consequently lower leaf number.

The absence of other significant differences on yield parameters, when low PPFs were used, do not agree with the results presented by other researchers working with lettuce in similar conditions. For instance, working at 60 µmol m^{-2} s^{-1} PPF intensity, Kobayashi, Amore and Lazaro (2013) reported significant differences in root and total plant dry weight, between fluorescent lamps and monochromatic R or B LEDs. Chen et al. (2014), using 133 µmol m^{-2} s^{-1}, found significant differences in shoot dry weight by comparing growth in R, B, R+B LEDs and fluorescent lamps. Chen et al. (2016) working with 135 µmol m^{-2} s^{-1} (105 µmol m^{-2} s^{-1} from W LEDs, supplemented with 30 µmol m^{-2} s^{-1} from other LED wavelengths), found significant differences in shoot and root fresh and dry weights.

These differences between ours and other researcher's results, may be related with different trial conditions (temperature, photoperiod, CO_2 concentration, plant nutrition) or different plant materials (cultivars), variables already identified by Snowden et al. (2016) as possible sources of variation, which contribute for the difficulty to compare results. It seems that not only PPF standardization is needed, similar actions are also required for other variables.

For treatments where higher PPFs were used (250 and 400 µmol m^{-2} s^{-1} for lettuce and 340 µmol m^{-2} s^{-1} for the other species), significant differences on yield parameters were already observed among the light spectra tested.

For lettuce, the highest fresh weight was achieved using 250 µmol m^{-2} s^{-1} PPF. Nevertheless, these results must be taken with precaution, as we believe that the semi-hydroponic growth system used, may have limited the uptake of water and nutrients by the plants, on situation of maximal request, not allowing the expression of all its full growth potential, when the 400 µmol m^{-2} s^{-1} PPF were used.

In fact, as it can be observed from figure 5, for this PPF and regardless the light spectra, the percentage of water on the leaves (H_2O %) was always significantly lower (3-5% less), when compared to any other PPF within the same spectrum, meaning that plants were in situation of water deficit on the 400 PPF treatment. The values of stomatal conductance, also being significantly reduced for all spectra, on this transition from 250 to 400 µmol m^{-2} s^{-1} PPF, seem to confirm this finding.

In situation of water deficit, the plant tends to close stomata in order to maintain its internal homeostatic equilibrium. So, the higher H_2O % on plants illuminated with R LEDs was probably related with the lower stomatal conductance these plants had, which in turn supports the highest values of fresh weight registered for those treatments.

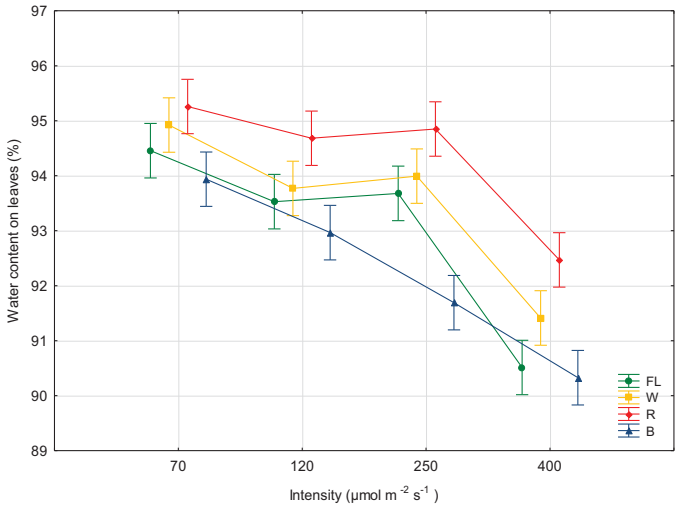

Figure 5. Behaviour of the water content on leaves of lettuce plants, growing under 4 light spectra and 4 light intensity regimes. Bars represent the 95% confidence intervals

Also from observation of figure 5, we can see on the B LED treatments a proportional reduction on H_2O % for each PPF increase. However, the high values of stomatal conductance registered, linked to a high photosynthetic rate, can justify the nonexistence of differences in the dray weight values among treatments, for each PPF tested.

Is possible to conclude that growth conditions linked to the highest PPF used were limiting plant growth potential, but probably did not encompass a stressful condition as Fv/Fm values bellow 0,8 were only observed for plants growing under R LEDs. The trend towards inadequate values for the Fv/Fm parameter in monochromatic R, was also observed by Son and Oh (2013). According to Fu et al. (2012) the relation Fv/Fm reflects the maximal photochemical efficiency of the active center of PSII in the dark. A greater Fv/Fm value results in higher light utilization efficiency and stronger ability of plants to adapt to low-light conditions. Under normal physiological conditions, the Fv/Fm values of the vast majority of C3 plants are between 0.8-0.84. When the Fv/Fm value of a plant is below this range, the plant is exposed to some environmental stress, such as drought, low or high temperature, or light stresses.

Concerning photosynthetic rates, those were alaso generally lower when red light was used, although significant differences among spectra only become evident for the highest PPFs. Working with cucumber, Hogewoning (2010), also obtained the lowest values of photosynthesis with monochromatic red. This author refers the existence of a "red light syndrome" associated to lower photosynthetic rates. Low levels of photosynthesis were in fact observed for several species subjected to 100% red monochromatic light, rice (Matsuda, Ohashi-Kaneko, Fujiwara, Goto, & Kurata, 2004), wheat (Goins, Yorio, Sanwo, & Brown, 1997), or radish (Yorio et al., 2001). Wang et al. (2016) also observed lower values of photosynthesis in 100% red, which were attributed to unresponsive gs and stated that photosynthetic performance of lettuce plants could be efficiently improved by increasing blue light fraction.

In on our trials, apart from the 100% R LED treatment, for all the other spectra, the photosynthetic rate continued its linear growth trend, observed on each PPF increase, from de lowest to the highest tested (70 to 400 μmol m^{-2} s^{-1}). So, before considering 250 μmol m^{-2} s^{-1} as the optimal PPF for lettuce development on controlled environmental growth conditions, data confirmation should be done for plants growing under higher PPFs, using production systems where availability of water and nutrients for the plants can be optimized.

Light intensity also affected significantly the relative chlorophyll content, which presented a growing trend on the four light sources, as light intensity increases. Cope, Snowden and Bugbee (2014), comparing PPFs of 200 and 500 μmol m^{-2} s^{-1}, also found for lettuce a relative chlorophyll (Chl) concentration consistently higher working with higher PPFs (500 μmol m^{-2} s^{-1}).

The same authors, working with different proportions of R and B light, reported an increase in Chl content as B light increased from 0% to about 30% and then a decrease with 92% B light. In our experiments, while using 100 % B light, the Chl content increased with the PPF increase, but, for the highest PPF tested, the Chl content was significantly lower for B and R LEDs, when compared with the broad light spectra of FL lamps and W LEDs. As stated by Cope et al. (2014), it seems that Chl synthesis is associated with phytochrome activity, but regulated by a complex interaction of phytochrome and cryptochromes, which is not fully understood.

Borowski et al. (2015) and Wang et al. (2016) observed that higher fractions of Chl in lettuce were achieved with the increasing of B light, and, by the contrary, in monochromatic R, the Chl content was significantly lower. In our trials the Chl content obtained with R LEDs, always presented values significant lower when compared with the other light spectra, inside the same light class intensity. It seems that the absence of blue and green wavelengths, for a given light intensity, significantly affects the content of Chl in the leaves, being these effect accentuated with the increase in light intensity. In fact, previous work have shown that blue deficiency was adverse to Chl biosynthesis in species like wheat (Tripathy & Brown, 1995), spinach (Matsuda, Ohashi-Kaneko, Fujiwara & Kurata, 2008), Rosa × hybrida (Terfa, Solhaug, Gislerød, Olsen & Torre, 2013), and cucumber (Hogewoning et al., 2010; Hernández & Kubota, 2016).

Apart from its specific effect on Chl content, light spectra affected plant development in general, being the most relevant aspect related with the plants growth behavior under W LEDs, with the performance of all species tested, never being inferior to the one achieved with the use of fluorescent tubes. A positive response of the plants to the light produced by W LEDs was already described for instance by Lin et al. (2013). This researchers, working with lettuce, growing under 210 μmol m^{-2} s^{-1} PPF, compared a mixture of RBW LEDs with a mixture of RB LEDs and verified that the fresh and dry weight of shoots were higher with RBW treatments. The authors assumed that an increase in plant growth with the addition of W light, can be related with its capacity to better penetrate the plant's canopy, providing more light for photosynthesis. Also Zhang et al. (2015), working with lettuce under conditions of commercial plant factory (200 μmol m^{-2} s^{-1} PPF above the canopy and supplementation of basal light at 40 μmol m^{-2} s^{-1}), reported that W LEDs were the most suitable for plant growth, being possible to increase the tradable fresh

weight and plant quality. This supports the idea that W LEDs can efficiently replace the traditional florescent tubes for plant growth on controlled environment and confirms Mitchell's (2015) thought, when he refers that after a quarter century of testing and development we are in fact rediscovering the value of white light.

But data from our trials also shown that the use of specific narrow wavelengths (R/B) can be of interest on some situations and that plants can efficiently adapt light receptors to the available wavelengths, in order to survive. In the lettuce trial, for instance, Fv/Fm values below 0.8 were registered, indicating somehow that the plants develop under stressful conditions while growing under 100% red light, but, the highest values of fresh and dray weight were also registered. Son and Oh (2013) also reported the highest fresh weight with the monochromatic R LED treatment, when compared lettuce growing under 100% R and different combinations of R and B light. Lee and Kim (2013) also working with lettuce and comparing fluorescent lamps with red and blue monochromatic LEDs, verified the same trend, fresh and dry weight increased in R and decreased in B, when compared to the fluorescent control.

However, morphological changes occurred at the leaf level, which significantly reduced the trade value of the plants. This morphological changes on leaf anatomy, characterized by elongated petioles and leaf curling, is also referred by Massa et al. (2008) or Hogewoning (2010), in situations of plant growth with monochromatic R light.

The absence of B light implies dysfunctions in the photosynthetic apparatus in particular the tissues between the veins. It is referred that an important difference between R and B light is the absence of cryptochrome and phototropin stimulation in pure R, whereas pure B does stimulate cryptochromes, phototropins and also phytochromes. It was conclude that adding a small amount of B light (7 μmol m^{-2} s^{-1}) these anomalies disappeared, indicating that B light has a quality effect on photosynthetic performance. Ouzounis, Rosenqvist and Ottosen (2015) also refer that plants cannot optimally develop with monochromatic R light, needing other wavelengths, like B and FR, to regulate other types of responses besides photosynthesis and biomass production.

5. Conclusions

LED light opened a new array of possibilities in order to understand plant growth behaviour under narrow band wavelengths, and, most researchers, are still looking for the best combination of R/B/G LED light in order to optimize production. Plants evolved during millions of years under sunlight, possessing different types of photoreceptors which respond to wavelengths from ultraviolet to infrared. Limiting wavelength availability on plant production systems, is in fact to limit the tools developed by the plants for light interception during their evolution. Besides, the use of broad light spectra under photosynthetic active radiation (PAR), has the advantage of reconciling the light needs of plants with the comfort for human vision. Also, the visual diagnosis of symptoms associated with plant nutritional deficiencies, or diseases, are easier to detect with white light than with monochromatic combinations of R/B/G LEDs.

Our results demonstrate that the use of white LEDs, having a broad spectrum that covers the PAR, can be a viable alternative for plant production under controlled environment conditions, efficiently replacing the traditional light sources. Also became evident from acquired data, that light intensity strongly affected plant yield and physiological parameters, being more important for biomass production and photosynthetic performance than light quality emitted by the different light sources. Nevertheless, the lack of standardization of trial parameters among researchers, like the PPFs, still makes difficult to compare results.

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Regulation of Growth and Carbohydrate Metabolism in Rice (*Oryza Sativa* L.) seedlings by Selenium and Sulphate

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Abstract

Selenium is an essential and also toxic trace element for organisms including plants. We studied the role of selenium (Na_2SeO_4) on growth and carbohydrate metabolism and its interaction with sulphate (Na_2SO_4) in rice (*Oryza sativa* L. cv. Satabdi) seedlings. Low concentration of selenium (2µM) showed stimulatory effect on growth as opposed to its higher concentration (50µM). Selenium was found to accumulate in a dose dependent linear pattern in the plant tissues. Exposure to selenate increased both reducing and non reducing sugar contents in the rice seedlings accompanied with an increase in the activities of sugar metabolizing enzymes like Sucrose Synthase (EC 2.4.1.13) and Sucrose Phosphate Synthase (EC 2.4.1.14). An increase in Starch Phosphorylase (EC 2.4.1.1) activity corresponded with the reduction in starch contents in the rice seedlings. Since Selenium is chemically analogous to sulphate, simultaneous application of sodium sulphate (10mM) and selenate (Na_2SeO_4) was found to ameliorate partially or totally all the tested parameters under selenate treatment alone resulting in alteration of growth and development of the test seedlings.

Keywords: Amelioration, growth, rice, selenium, sugar metabolism, sulphate

Abbreviations: β-ME - beta mercaptoethanol, cv.- cultivar, DNSA- 3,5-dinitrosalicylic acid, dw - dry weight, DTT- dithiothreitol, EDTA- ethylenediamine tetraacetic acid, fw- fresh weight, HEPES- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, KI- potassium iodide, PFD- photon flux density, PMSF- phenyl methyl sulphonylfluoride, S- Sodium sulphate salt, Se- Sodium selenate salt, SE- standard error, SPS- sucrose phosphate synthase, SS- sucrose synthase, TCA- trichloroacetic acid ,UDP- uridine-di-phosphate.

1. Introduction

The present investigation was undertaken to widely examine the influence of selenium singly as well as in combination with sulphate on the metabolic status of sugar, starch and different sugar metabolizing enzymes in germinating rice (*Oryza sativa* L. cv. Satabdi) seedlings.

1.1 Literature Review

Selenium, a chalcogen of sulphur, is a Group VI A, Period 4 trace element of oxygen sulphur family according to the periodic table (Bodnar, Konieczka & Namiesnik, 2012). It has a significant role to play in the metabolism of both animals and plants. Selenium alters oxidative stress, DNA methylation, DNA repair, apoptosis, cell proliferation, carcinogen metabolism, hormone production and immune function in different animal systems (Dinkova-Kostova 2013; Hatfield, Tsuji, Carlson & Gladyshev, 2014). In plants, selenium does a paradigm shift between playing the role of an antioxidant or a pro-oxidant at specific concentration (Pennanen, Xue & Hartikainen 2002). Present interest in selenium is focused on health benefits using biofortified plants with high selenium contents as a source of cancer preventive selenium compounds (Zhao & McGrath 2009; Dinkova-Kostova 2013).

In plants, selenium at low concentration enhances growth and ability to withstand stress (Hartikainen et al., 2000; Sun et al., 2010) whereas it becomes toxic at higher concentrations (Mroczek-Zdyrska & Wójcik 2012). In electrochemical series, selenium being physico-chemically similar to sulphur acts as a chalcogen (Bodnar et al., 2012). According to Missana et al. (2009) and Winkle et al. (2015), among the two inorganic forms of selenium, selenate (SeO_4^{2-}) is more bioavailable than selenite (SeO_3^{2-}) from anthropogenic sources. In plants, selenate

(SeO_4^{2-}) is actively transported through sulfate transporters as it shows chemical similartity with sulfate ion (Dumont et al., 2006, El Kassis et al., 2007; Gigolashvili & Kopriva, 2014).Therefore, the presence of sulfate ion can influence uptake of selenium in plant tissue as observed in *Astragalus* , *Aradidopsis, Brassica* and *Stanley* species by Sors et al. (2005), El Kassis et al. (2007), Cappa et al. (2014) and Schiavon et al. (2015) respectively.

In growing plant tissues, accumulation of sugar occurs to counteract stressful environment through osmotic alterations (Mishra & Dubey 2008; Rosa et al., 2009). The primary end products of photosynthesis sucrose is one of the major form of translocated carbon (Zhou et al., 2002) whereas starch comprises the temporary reserve form of carbon which gets finally stored in the grains (Zeeman et al., 2004). The enzyme Sucrose Phosphate Synthase catalyses sucrose synthesis in the plant tissues whereas Sucrose Synthase, present in cytosol, helps in sucrose breakdown in vivo and translocating the assimilates to diverse pathways in plant storage cells (Huber & Huber, 1996). Yang et al. (2001) reported that in plants, Starch Phosphorylase hydrolyzes starch by incorporating phosphate at the non-reducing end.

2. Material and Methods

2.1 Plant Materials and Selenium Treatments

Rice (*Oryza sativa* L. cv. Satabdi) seeds were obtained from the State Rice Research Institute, Chinsurah, Hooghly, West Bengal, India. The seeds were surface sterilized with sodium hypochlorite (0.5 %) for 15 mins. and then washed thoroughly in distilled water. A batch of 100 seeds were arranged in petri dishes with filter papers containing 20 mL sterile water (as control). Different concentrations of sodium selenate (Na_2SeO_4) purchased from Loba-Chemie, India or selenate in combination with sodium sulphate (Na_2SO_4) purchased from Merck, India were applied to the experimental sets. The selenate concentrations used in the present experiment were similar to selenate concentrations found in different field conditions.

For 48 hours seeds were placed in a germinator (30 ± 2°C) followed by exposure to 16 hours photoperiod and 75% relative humidity. The rice seedlings were further grown in modified Hoagland solution prepared with respective selenate and sulphate concentrations which were replaced on every alternate day for twenty-one days. After twenty-one days treatment plants were collected, washed properly, root and shoot were separated and either used as fresh material or stored in -80^0C for the following experiments. All the experiments were conducted in a randomized design and repeated thrice.

2.2 Morphological Studies

Following twenty one days of treatment, the effects of selenate singly and with sulphate were observed on test seedlings. Length of root and shoot of rice seedlings as morphological data were recorded and the root tolerance index (RTI) and shoot tolerance index (STI) were calculated from the root and shoot length data respectively.

2.3. Extraction and Estimation of Selenium Content

Total selenium contents were measured from acid digested 1g dried root and shoot samples of rice. The dried samples were digested in a Microwave Digestor using 7ml of HNO3 (65%), 5ml HCl and 2ml of H_2O_2 for about 60 min. After digestion, Selenium contents were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) iCPA 6000 series (Thermo Scientific) using a standard curve prepared from known concentrations of selenium solutions and expressed as mg kg^{-1} dw.

2.4 Estimation of Starch and Sugar Content

Total soluble sugar was assayed according to Dubois et al. (1956). 1g root and shoot samples were crushed with 80% ethanol and centrifugation was done for 20 minutes at 2000 rpm. 5% phenol (0.05 ml) and sulphuric acid (98%) were mixed with 1 ml supernatant followed by 20 min incubation in water bath at 30ºC. Finally, OD values of the yellow orange colour solution were taken in Hitachi-2000 spectrophotometer at 490 nm. Standard curve of glucose was prepared using known concentrations of glucose (Nelson 1944) and total soluble sugar contents was calculated accordingly. Total soluble sugar was expressed in terms of mg g^{-1} fw.

Reducing sugar was quantified according to the method of Miller (1972). 1g plant samples were homogenized in 80% ethanol followed by 20 minutes centrifugation at 2000 rpm. To 1 ml of alcoholic supernatant, DNSA was mixed and boiled for 5 minutes. OD was taken at 515 nm in Hitachi-2000 spectrophotometer. Glucose concentration was quantified using a standard curve of glucose and the amount of reducing sugar was expressed as mg g^{-1} fw. Amount of non-reducing sugar was calculated by substracting the value of reducing sugar from the value of total sugar.

Quantification of starch was carried out according to the method of McCready et al.(1950).The remaining mass, gained after centrifugation for the extraction of total soluble sugar was again suspended in distilled H_2O and

perchloric acid was added followed by centrifugation at 2000 rpm for 20 minutes. The supernatant was then collected, poured in conical flasks and the final volume was made upto 100 ml by addition of distilled water. Starch content was measured from 1.0 ml of filtrate following the same protocol of total soluble sugar. Starch content was quantified in terms of glucose and factor 0.9 was applied for the conversion of glucose to starch. Amount of starch was expressed in terms of mg g^{-1} fw.

2.5 Preparation of Extracts and Enzyme Assays

Starch phosphorylase activity was determined according to Dubey and Singh (1999). Crushing was done in 50 mM citrate buffer (pH 6.0) containing β-ME (5 mM), EDTA (1 mM), PMSF (1 mM) and centrifugation was carried out for 20 min at 10000 rpm at 40^0C. The assay mixture constituted of 50 mM citrate buffer (pH 6.0), 0.1mM glucose-1-phosphate, 5% soluble starch (w/v), and enzyme extract to make the final volume upto 4.0 ml. 5% TCA was added after 10 minutes to terminate the reaction. Phosphorous contents were measured after centrifugation according to the method of Fiske and Subbarow (1925). Enzymatic activity was expressed as μmol of Pi liberated g^{-1} protein min^{-1}.

For the assay of Sucrose Phosphate Synthase (SPS) and Sucrose Synthase (SS), the plant tissues were homogenized following the method of Hubbard et al. (1989) and assayed according to Miron and Schaffer (1991). Plant samples were crushed in 50 mM HEPES-NaOH buffer (pH 7.5) containing EDTA (1 mM), $MgCl_2$ (5 mM), 0.05% (v/v) Triton X-100 and DTT (2.5 mM) followed by centrifugation at 10000 rpm for 10 min. Assay mixture for SPS constitute of enzyme extract, 50 mM HEPES-NaOH buffer (pH 7.5), fructose-6-phosphate (25 mM), glucose-6-phosphate (25 mM), $MgCl_2$ (15 mM), UDP-glucose (25 mM).The reaction was stopped after 30 min incubation at 37ºC by addition of 30% KOH. Reaction mixture of sucrose synthase assay was like sucrose phosphate synthase except that it required fructose (25mM) instead of fructose-6-phosphate and glucose-6-phosphate was absent. Sucrose hydrolysed or formed by SS or SPS catalysed reaction was measured according to Vassey et al. (1991). The enzyme activities were expressed as nmol sucrose hydrolysed or formed g^{-1} protein min^{-1} respectively.

2.6 Protein Estimation

For all enzyme preparations the concentrations of protein were measured according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.7 Statistical Analysis

The experiments were carried out in a completely randomized design (CRD) with three repeats, each treatment comprising a single petridish containing 100 seeds. The data and significant differences among the mean values were compared by descriptive statistics (±SE) followed by Student's't'-test. The alphabet 'a' indicates high statistical significance at $P \leq 0.05$ as compared to water control.

3. Results

3.1 Influence on Seedling Growth

Exposure of rice seedlings to different concentrations of selenate showed both stimulatory and inhibitory effects on elongation of root and shoot lengths. Maximum stimulation occurred in 2 μM selenate treated rice seedlings which were about 36% in root and about 31% in shoot over water control.Thereafter, a sharp decline were observed in growth of rice seedlings which were about 34% and 67% in root and about 15% and 51% in shoot under 20 μM and 50 μM selenate treatment respectively (Figure 1). The root and shoot tolerance index also concomitantly reduced with increase in concentrations of selenate. Roots were found to be more affected than shoot in the test cultivar.

Joint application of sulphate (10 mM) along with selenate altered the effect caused by selenate alone and induced stimulation in both root and shoot length. The root and shoot length almost doubled under combined application of 2 μM selenate and sulphate whereas the inhibitory effect on growth were narrowed down to a maximum of about 33% in root and by about 28% in shoot over water control. Similarly, joint application of said concentrations of selenate with 10mM sulphate increased the RTI and STI respectively in the test cultivar (Figure 2).

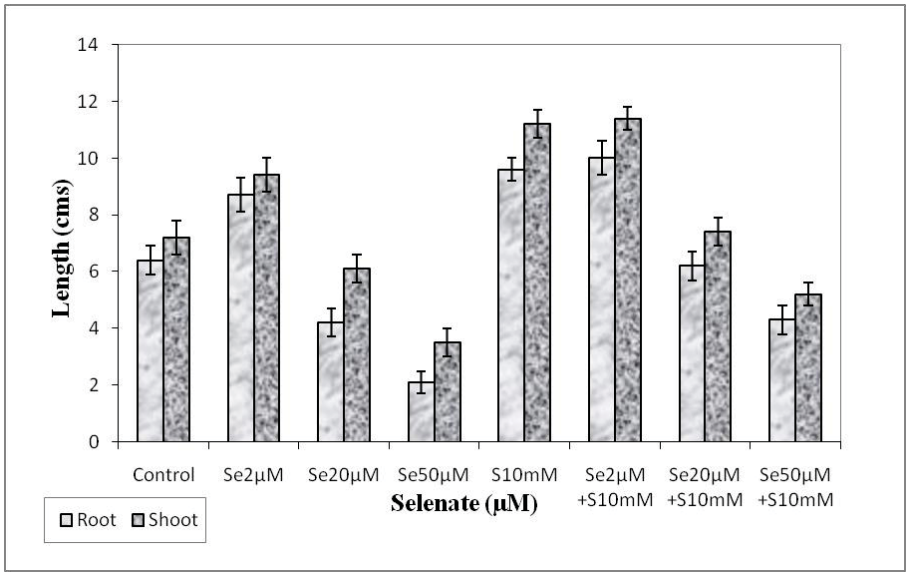

Figure1. Effect of selenate and/or sulfate on shoot and root lengths in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each bar is the mean ± SE with three repeats

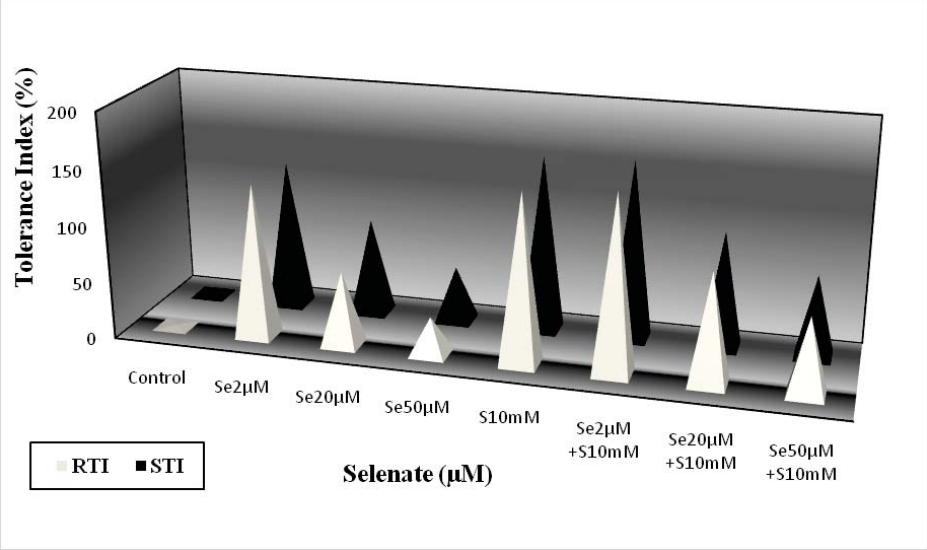

Figure2. Effect of selenate and/or sulfate on RTI and STI in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each bar is the mean ± SE with three repeats

3.2 Influence on Selenium Contents

The selenium contents were negligible in root and shoot of rice seedlings grown only in water (control) but it increased markedly with the increasing amount of selenium added to the test solutions (Figure 3). In roots, maximum accumulation of Se took place under 50 µM selenate treatment that was about 5 times more than that found in shoots at the same concentration of selenium. Joint application of selenate and sulphate (10 mM) showed varied responses on selenium uptake. In root, the selenium contents increased under 2 µM selenium and 10mM sulphate treatment whereas it decreased in 50 µM selenium plus sulphate treated test samples compared to the level under same concentration of selenate alone. In shoot, 10mM sulphate applied jointly with 2 µM, 20 µM and 50 µM selenium showed a decline in selenium uptake over water control.

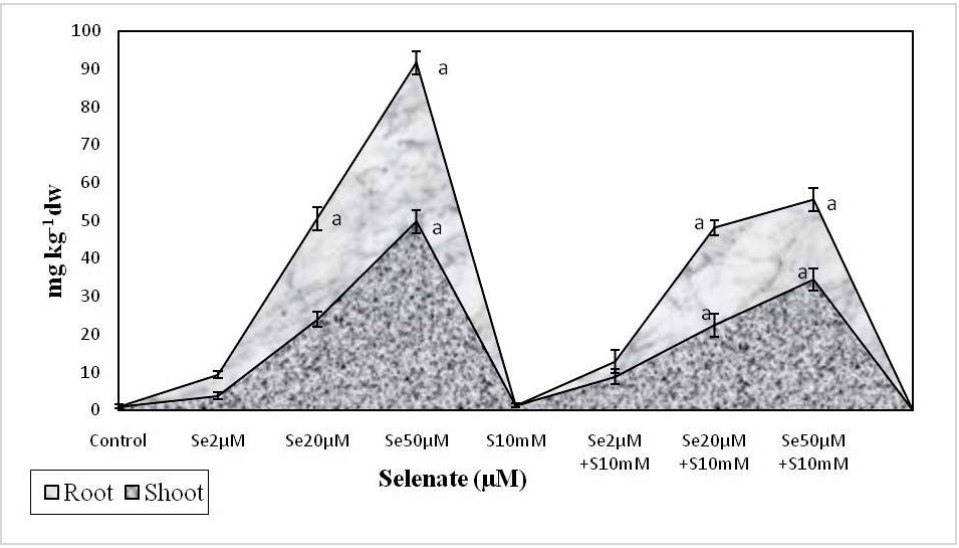

Figure 3. Effect of selenate and/or sulfate on selenium content in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each bar is the mean ± SE with three repeats. The alphabet 'a' indicates high statistical significance at $P \leq 0.05$ as compared to water control

3.3 Influence on Starch Contents

In both root and shoot of the test seedlings, the starch contents decreased with increasing selenate treatment although it was higher with respect to water control. The starch level registered a decline on an average of about 18% in roots and about 8% in shoots of the treated rice seedlings (Figure 4).Joint application of selenate with 10mM sulphate altered the effect caused by selenate alone in the test cultivar. Co-application of 10mM sulphate along with 20µM and 50µM selenate increased starch contents on an average by about 14% in roots and by about 10% in shoot of rice seedlings respectively over water control (Figure 4).

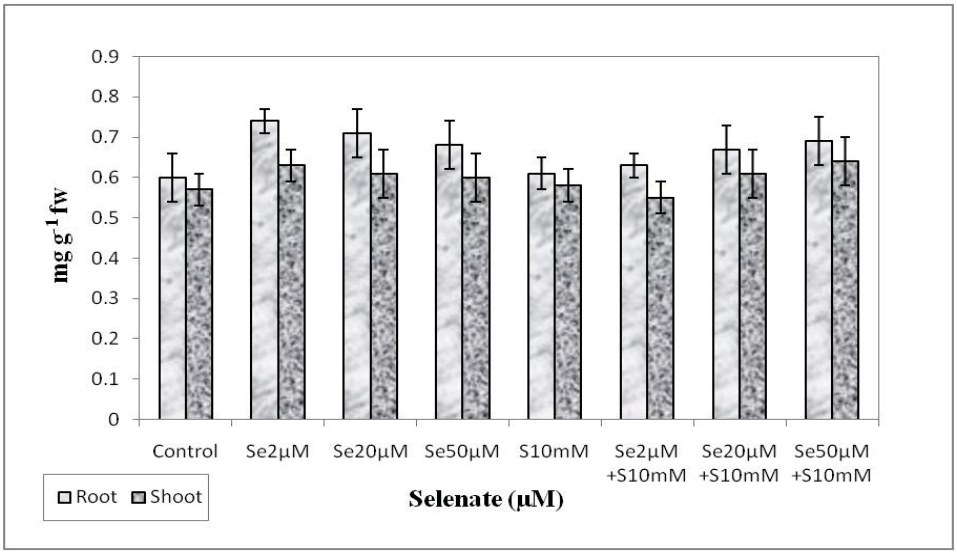

Figure 4. Effect of selenate and/or sulfate on starch contents in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each data point is the mean ± SE with three repeats

3.4 Influence on Reducing Sugar Contents

The reducing sugar contents increased in both roots and shoots of the test cultivar with increase in selenate treatment. The reducing sugar contents were stimulated by about 14%,23% and 28% in roots and by about 6%,22% and 35% in shoots of rice seedlings under 2 µM, 20µM and 50µM selenate treatment respectively over

water control (Figure 5). Maximum inhibition were recorded in rice seedlings treated with 50µM selenate and sulphate (10 mM) where the reducing sugar level decreased by about 11% in roots and about 21% in shoots of rice seedlings with respect to water control (Figure 5).

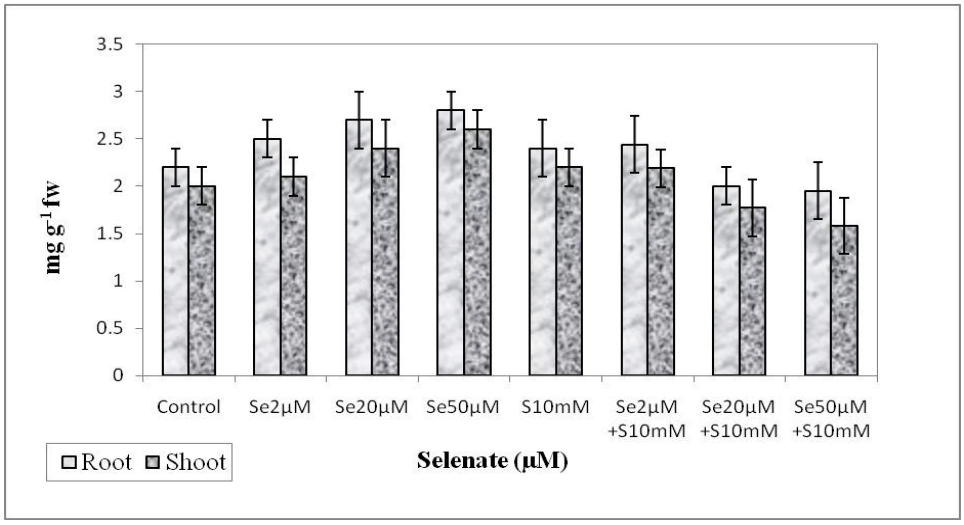

Figure 5. Effect of selenate and/or sulfate on reducing sugar contents in shoots of rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each data point is the mean ± SE with three repeats

3.5 Influence on Non-Reducing Sugar Content

The level of non reducing sugar were enhanced by about 11%,23% and 28% in roots and 13%,19% and 32% in shoots of 2 µM, 20µM and 50µM selenate treated rice seedlings respectively over water control (Figure 6).Application of said concentrations of selenate in combination with sulphate (10 mM) decreased the level of non reducing sugar contents by about 3%,17% and 21% in roots and by about 4%, 10% and 15% in shoots of test seedlings respectively over water control (Figure 6).

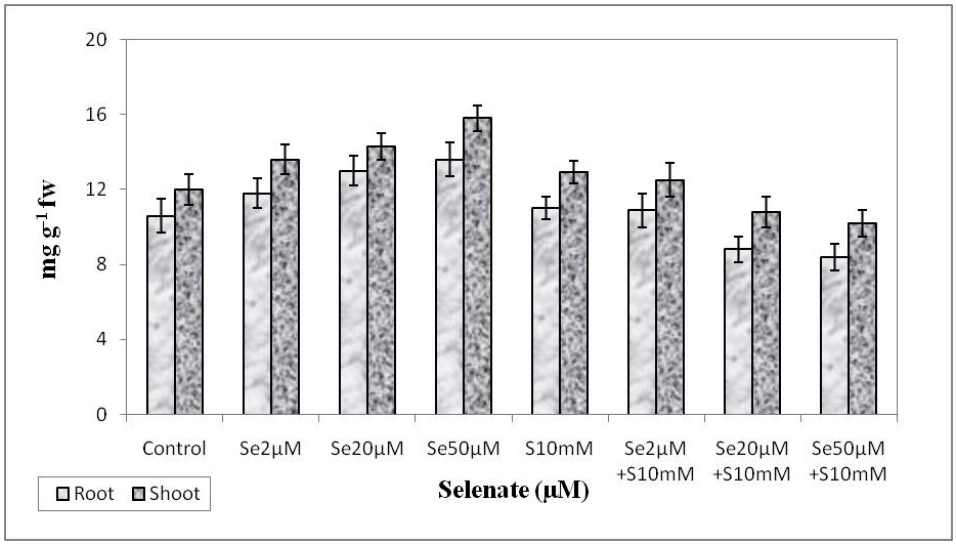

Figure 6. Effect of selenate and/or sulfate on non reducing sugar contents in shoots of rice (cv. Satabdi) seedlings. The data were recorded from 21days old seedlings. Each data point is the mean ± SE with three repeats

3.6. Influence on Sucrose Synthase Activity

The activity of sucrose synthase (SS) was increased in both roots and shoots of selenate treated rice seedlings. The enzyme activity increased on an average by about 22% in roots and 21% in shoots of the test samples compared to water control (Figure 7). Application of higher concentrations of selenate with 10mM sulphate

simultaneously, reduced the enzyme activity to a maximum of about 12% in roots and about 16% in shoots of the test cultivar with respect to selenate treatment alone.

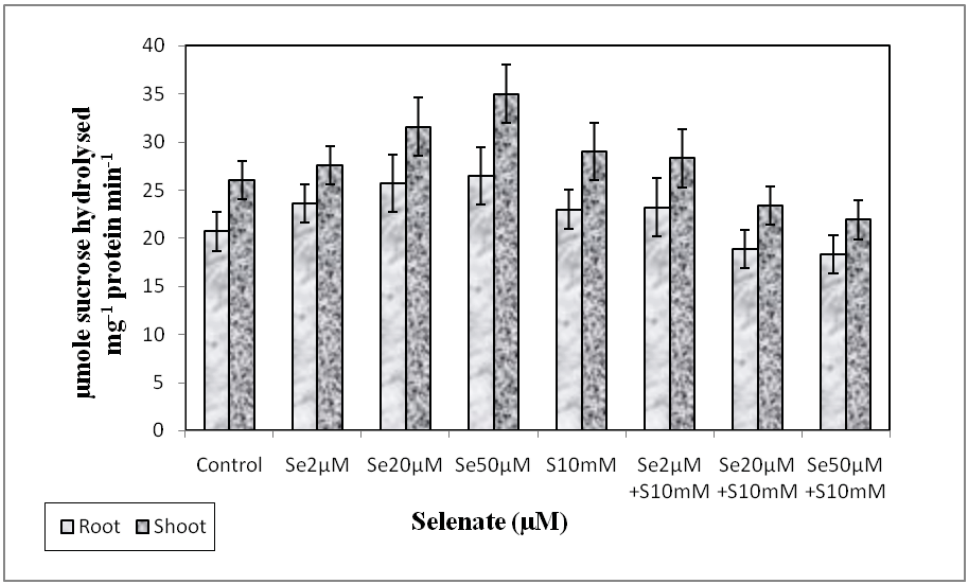

Figure 7. Effect of selenate and/or sulfate on sucrose synthase activity in rice (cv. Satabdi) seedlings. The data were recorded from 21days old seedlings. Each data point is the mean ± SE with three repeats

3.7 Influence on Sucrose Phosphate Synthase Activity

The sucrose phosphate synthase activity was stimulated in both root and shoot of test cultivar under selenate treatment (Figure 8). The enzyme activity recorded a linear increment of about 12%, 27%and 32% in roots and about 15%, 21%and 33% in shoots of 2 µM ,20 µM and 50 µM selenate treated rice seedlings respectively. Joint application of selenate with 10mM sulphate reversed the effect caused by selenate alone and reduced the enzyme activity on an average by about 12% in roots and by about 15% in shoots of the test samples with respect to water control.

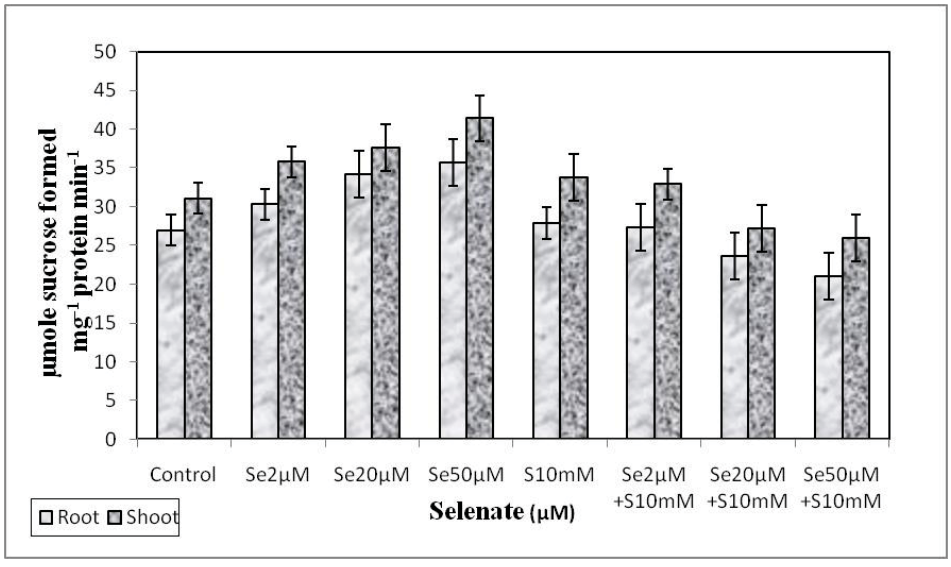

Figure 8. Effect of selenate and/or sulfate on sucrose phosphate synthase activity in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each data point is the mean ± SE with three repeats

3.8 Influence on Starch Phosphorylase Activity

Rice seedlings showed both stimulatory and inhibitory effects on starch phosphorylase (SP) activity due to

selenate treatment .The effect was less pronounced in shoot than root of test seedlings. Initially the enzyme activity declined by about 10%, on an average, both in root and shoot of test seedlings under 2 μM selenate treatment. Thereafter, the enzyme activity increased considerably to a maximum of about 32% in root tissue and 21% in shoot tissue of rice seedlings under 50 μM selenate treatment compared to water control (Figure 9).Co-application of 2 μM selenate and sulphate inhibited the promotive effect on the enzyme activity by about 6%, on an average, in root and shoot of the test cultivar. The enzyme activity decreased by about 25% in root and about 8% in shoot, on an average, under combined application of higher concentrations of selenate with 10mM sulphate.

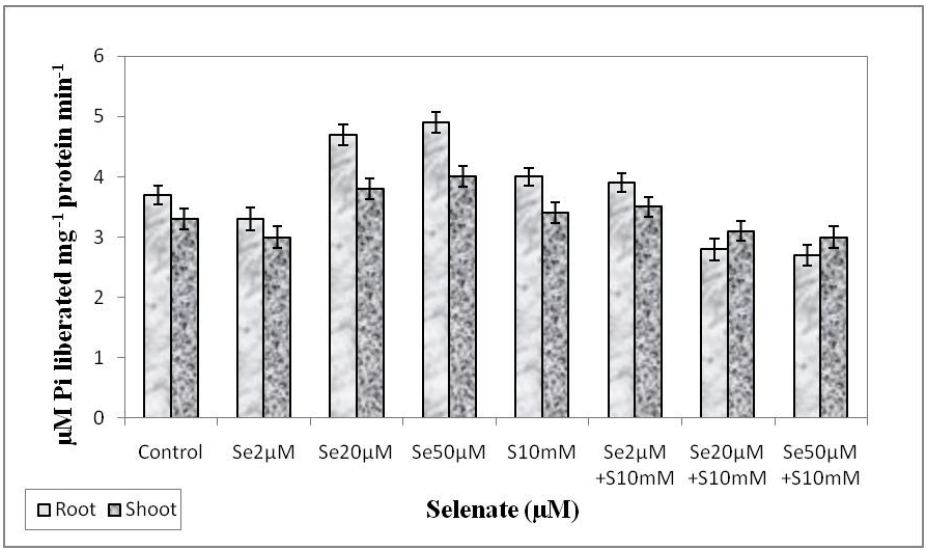

Figure 9. Effect of selenate and/or sulfate on starch phosphorylase activity in rice (cv. Satabdi) seedlings. The data were recorded from 21 days old seedlings. Each data point is the mean ± SE with three repeats

4. Discussion

4.1 Influence of Selenate and Sulphate on Selenate Uptake and Growth of Seedlings

Selenium (Se) exposure showed variable influence and altered normal growth and development in the seedlings of rice cultivar (cv.Satabdi) under selenate treatment. Low concentration of selenate (2 μM) showed stimulatory effect on growth in comparison to higher concentrations of selenium (≥ 20 μM) which inhibited the development of the rice seedlings. Roots were most affected than shoots and were found to be severely injured with browning at the apical tissue region. Similar observations were noted by Khattab et al. (2004) and Chu et al. (2013) on other plant species. The level of selenium increased linearly in a dose-dependent manner with increasing concentrations of selenate in the test seedlings whereas joint application of selenium and sulphate at concentrations higher than 2 μM selenate, reduced the intake of selenium but induced a dose-dependent increase in sulphate accumulation in the plant tissue. Similar increase in sulphate accumulation on selenium supplementation was reported in shoots of many plants including *Brassica oleracea* (Kopsell et al., 2000). Our results are also supported by White et al. (2004) who observed similar alterations occuring in *Arabidopsis thaliana*.

4.2 Effect of Selenate and Sulphate on Carbohydrate Metabolism

The flow of photosynthetic assimilates from source to sink organ helps to regulate partitioning of dry matter in plants which is important during plant growth and development. It is also considered as a limiting factor in crop yield. Atmospheric carbon is photosynthetically fixed in the form of sucrose and starch at the end of photosynthesis. Starch acts as a temporary storage form of fixed carbon in the chloroplast and is finally stored in the cereal grains. Sucrose is the primary transportable sugar in plant system. Presence of enhanced quantities of soluble reducing and non-reducing sugars in the test seedlings coincide with the activity of Sucrose Synthase and Sucrose Phosphate Synthase. Such enhancement in sugar contents might help to increase cellular respiration in order to counteract the toxic effects of high selenium concentrations in the root and shoot of the test seedlings. Previously, it had been demonstrated by Quick et al. (1989), Dubey and Singh (1999) and Devi et al. (2007) that water, salinity and cadmium stress led to increment in soluble sugar contents. According to Couee et al. (2006)

abiotic stresses also seems to provoke accumulation of soluble sugars as a counteractive way to ensure the maintainance of homeostasis in the cells . The present study also showed a decrease in starch contents under high selenate concentrations which may occur due to starch degradation, or reduced synthesis of starch in order to counteract selenium stress. Similar results were documented by Rahoui et al. (2008) in cadmium treated *Vicia faba* seedlings. Starch phosphorylase catalyses starch hydrolysis by incorporating phosphate (Salisbury & Ross, 1991). Increment of starch hydrolysing enzyme, starch phosphorylase activity is correlated with the decrease in starch contents as observed in the test seedlings. Sucrose Synthase (SS) has a vital role in sucrose metabolism in plants. Sucrose synthase is a cytosolic enzyme that regulates synthesis and breakdown of sucrose in plants (Zheng et al. 2011). Sucrose phosphate Synthase (SPS) regulates carbon flux in a reversible reaction forming sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate during sucrose formation in higher plants. In the study an increase in Sucrose Synthase and Sucrose Phosphate Synthase activities were recorded both in root and shoot of rice seedlings treated with high concentrations of selenium. Similar increase in activity of Sucrose Synthase was observed by Verma & Dubey (2001) in rice seedlings under cadmium toxicity. According to Yang et al. (2001) enhanced activity of said enzymes related to sucrose metabolism may have positive effect in adaptation of the rice seedlings under selenium stressed condition by osmotic adjustment, thus shielding the biomolecules and membranes from dehydration. When the test seedlings were further treated jointly with high concentrations of selenate (>2 μM) and sulphate (10 mM), the activity of the enzymes were found to be partially or completely altered.

Majority of mankind on earth consumes rice (*Oryza sativa* L.).Rice is also considered as the most important staple food crop in India. Rice is the second most efficient selenium accumulator plant among the cereal crops and thus possesses the capability to become an important source of dietary selenium (Poblaciones et al. 2014). In order to produce food products biofortified with selenium, it becomes necessary to choose sustainable crop varieties that accumulates Se at a moderate concentration in their edible parts as discussed by Mayer et al.(2008), Liu et al. (2011),Yin and Yuan (2012) and Wu et al. (2015). Selenate is analogous to sulphate. Therefore, external application of sulphate along with selenate to rice seedlings may help to overcome the detrimental effects caused by high concentrations of selenium which is evident from our investigations. The test results also indicate that the role of selenium and sulphate are complex in the rice growth system. Therefore, in order to produce Se-enriched rice, it is important to comprehend the interactions that occurs between selenium and sulphate in the plant system at all levels. Otherwise, it may result in the entry of excessive selenium into the food chain, consequently injuring human health.

5. Conclusion

The present study on role of selenium on carbohydrate metabolism in rice (*Oryza sativa* l.) cv. Satabdi seedlings and its interaction with sulphate is of significance as it is one of the few reports on selenium sulphur interaction in rice plants available to the best of our knowledge. Since supplementation of rice seedlings with sodium selenate increased selenium contents in the test cultivar and its toxic level was regulated on addition of sodium sulphate, this might provide an efficient and effective way to supervise selenium concentrations during biofortification in cereal plants. Our investigation is the first step towards understanding the physiological and biochemical interactions involved in the regulation of selenium intake by sulphate in rice seedlings and more research on selenate sulphate relationship is required to develop a selenium biofortified cereal crop which may serve as a sustainable and economic dietary source of selenium in the environment.

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