The Impact of Climate Change on the Symbiosis between the Dark Septate Endophytic Fungi and Koshihikari Rice Plant

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Abstract—**Rice production in South East Asia is under the threat of climate change such as global warming, which is projected to increase 2 °C or more in the late- 20th -Century (IPCC AR5). In that case, endophytes can be used as the natural-based adaptation tools since they are found obviously in different ecosystems. However, symbiosis activity between the endophytes and their host plant under high temperature is not well known, requiring more elucidation. The purpose of this research is to clarify the symbiotic capacity of endophytic fungi in the early stage of rice growth and to evaluate the impact of high temperature on rice-endophytic symbiosis through the observation of the fungi present in the roots. The symbiosis activity between the Dark Septate Endophytes (DSE) and Koshihikari rice plant under high temperature was tested by comparing *Cladophialophora chaetospira (OGR3), Meliniomyces variabilis (J_1PC_1), Phialocephala fortinii ($LtPE_2$), Veronaeopsis simplex (Y34)* and without colonization under continuous high temperature of 35 °C. Root colonization was checked under the microscope and the plant growth parameters such as root/shoot ratio, shoot high, etc., were measured. The colonization capacity of DSE fungi in the roots of rice plant was demonstrated for all the selected species at 35 °C. Generally, the root/shoot ratio was increased in all treatments with DSE. However, the root/shoot ration of treatment with *Cladophialophora chaetospira (OGR3) and Veronaeopsis simplex (Y34)* were highest among the four treatments. Therefore, the selected dark septate endophytic fungi have potential to be used as biofertilizers.**

Index Terms—**symbiosis, dark septate endophytic fungi, rice plant, climate change**

I. INTRODUCTION

Rice is the principle staple food for the half of the world's population, especially for the Southeast Asia, where, rice production reflects the main economy and livelihood for millions of people [1], [2]. The harvested rice in the Southeast Asia accounts for 30 percent of the world harvest [3]. However, the increasing occurrence of severe weather including heatwaves and precipitation events due to climate change are threatening rice production and productivity in the region [4]. The alteration in precipitation pattern under the climate change will add more pressure on rice production since 45 percent of the rice area of Southeast Asia is irrigated [5]. Under these circumstances, plant roots play the main roles in maintaining resistant capacity to biotic and abiotic stress by forming a symbiotic relationship with endophytic fungi. Among various root endophytes, Dark Septate Endophytes (DSE) are found in roots of more than 600 plant species, being ubiquitously distributed in the northern temperate zone (Grünig *et al.*, 2004), artic and alpine ecosystem (Haselwandter, 1981) [6], [7]. The endophytic symbionts facilitates plant nutrient uptake improvement, root pathogen and adverse environmental tolerance [8]. Despite its benefits for plant performance, the behavior of DSE fungi on rice roots is poorly characterized.

The genus *Cladophialophora chaetospira (OGR3)* is a novel DSE can be found in several along the Nothern Hemisphere (Narisawa *et al.* 2007) [9]. This species colonizes non-mycorrhiza roots of many species in the natural ecosystem, especially under nutrient-stressed condition so it is expected to improve plant growth under climate change negative impacts [10]. While *Meliniomyces variabilis (J_1PC_1)* is a co-associated fungi colonizing blueberry hair roots to enhance the plant shoot and root growth (Vohnik *et al.*, 2013); *Phialocephala fortinii ($LtPE_2$)* is one of the most abundant DSE species in European forest stand, effectively suppressing verticillium wilt in Chinese cabbage, increasing shoot and root dry weight of *Gnaphalium norvegicum* (reviewed by Grünig *et al.*, 2008). *Veronaeopsis simplex (Y34)* species is able to control disease caused by *Fusarium oxysporum* in Chinese cabbage (Khastini *et al.*, 2012; Guo *et al.*, 2018). [11]-[13].

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"Koshihikari" rice is well-known cultivar in western and central Japan (Takeuchi *et al.*, 2006), being a pride of Japanese due to the good flavor of its grain (Wasaka *et al.*, 2007). This rice species requires cool-temperature at the booting stage (Takeuchi *et al.*, 2006) [14]. However, the meteorological research projects an increase of one or two degrees all over Japan in 2081 to 2100, which may cause threats on the rice productivity and growth, [15]. Therefore, the inoculation of four above endophyte fungus in rice roots is promising to enhance resistant capacity of plant in coping with water stress and soil nutrient deficiency due to climate change impacts. The research implementation aims at i) elucidating the symbiotic capacity of endophytic fungi in early stage of rice growth; ii) evaluating the impact of high temperature on rice-endophytic symbiosis through the observation of fungi presence in the roots and iii) proposing possibility to apply dark septate endophytic fungi as biofertilizer.

Accordingly, one experiment was conducted with "Koshihikari" rice to test two hypotheses: i) the selected Dark Septate Endophytic (DSE) fungi can be well colonized with temperate rice plant under high continuous temperature, ii) if the selected DSE can colonize with temperate rice plant under high temperature, they may promote plant growth under high temperature and thus they have potential to be used as biofertilizer. Briefly, four replicates of singly endophyte inoculated rice was set up at College of Agriculture, Ibaraki University, Japan to compare with rice growth without endophytic symbiosis (control).

II. Materials and Methodology

A. Experiment Design

Koshihikari rice seeds were selected for the experiment as this is the most popular cultivar in Japan.

Rice Seed Germination: Seeds were sterilized by vortex with 70% ethanol for 2min, followed by 1% sodium hypocloride for 5min and then washed with sterile distilled water for 3 times, 5min each. After that, the seeds were immersed into the sterile distilled water using the 50ml conical laboratory tube and incubated at room temperature (19 ℃) for 2 days. After seed sterilization, the sterilized rice seeds were air dried in a laminar air flow cabinet for 30 min and transferred to the petri dishes containing water agar medium (500ml WA medium: agar powder 5g and sterile distilled water 500ml) for germination and placed in 30 ℃ incubator for one day.

Transplantation: The experiment was conducted with 5 treatments: Koshihikari rice plant without endophytic symbiosis (control), with *Cladophialophora chaetospira (OGR3), Meliniomyces variabilis (J_1PC_1), Phialocephala fortinii ($LtPE_2$), Veronaeopsis simplex (Y34)*. Each treatment was replicated 5 times.

In the control treatment, the germinated rice seeds were transplanted into petri dishes of oat meal medium which was mixed with other mineral and nitrogen sources ($MgSO_4$, H_2O, KH_2PO_4, $NaNO_3$, Nature Aid (Sakata-no-tane), powdered oat meal and agar for plant cultivation). For DSE fungi treatment, the germinated rice seed was transplanted into the petri dishes of oat meal medium. Then, each species of DSE fungi maintained in the petri dishes of 50% Corn Meal Malt Medium (derived from the culture collection of the laboratory of Microbial Ecology, Ibaraki University) was excised from an edge of an actively growing colony on culture medium into 5mm diameter circular shape by using autoclaved cork borer and placed near the root of the germinated rice seed. Then the open petri dishes were enclosed into the autoclaved plant culture PC square jar and transferred to the plant growth chamber of 35 ℃ for both day and night temperature.

B. Parameter of Research

Root colonization: Toluidine blue or TBO stain method was used for staining the roots and root colonization was checked by observing the morphology of DSE colonized blue stained roots under microscope.

After 10 days of transplantation, root and shoot length were measured with the ruler (cm). By using the measuring cup (ml), root volume was measured by inserting the root pieces into the cup and measure the volume difference before and after inserting the root.

Root dry weight was measure by inserting the fresh root into the oven for 24 hour at 70 ℃ and the root/shoot ratio was obtained by dividing the dry weight of the root by the dry weight of the shoot.

C. Bacteria Control

Since bacteria appeared after 24hr of transplantation. Then, the bacteria control was conducted in another experiment by adding 2% streptomycin into the oat meal medium for bacteria control, based on the preliminary test.

However, due to the limitation of stocks, the bacteria control was only applied for three treatments of Koshhikari Rice seed with *Cladophialophora chaetospira (OGR3), Meliniomyces variabilis (J1PC1), Veronaeopsis simplex (Y34),* respectively.

The drying time of the sterilized rice seeds was also changed into 24 hr instead of 30 min in order to prevent bacteria contamination from the vapor.

III. Results and Discussion

A. Colonization Capacity of DSE Fungi in Koshihikari Rice Roots in the Presence of Bacteria

Identification of bacteria was performed in order to verify the origin of bacteria. The percent identify of the DNA sequence of the selected three isolates (control, Y34, OGR3) based on the NCBI BLAST database is about 96% similarity with the target sequence (Table I). Therefore, the bacteria appeared in the first experiment can be a seed-originated species.

TABLE I. THE PERCENT IDENTIFY OF THE DNA SEQUENCE FROM THE SELECTED THREE ISOLATES OF THE FIRST EXPERIMENT BASED ON NCBI BLAST DATABASE

Description	Max Score	Total Score	Query Cover	E value	Per. Identity	Accession
Leclercia adecarboxylata strain L16 16S ribosomal RNA gene, partial sequence	1144	1481	89%	0.0	96.59%	KT937143.1
Uncultured bacterium partial 16S rRNA gene, clone SICC390_N11D2_16S_B	1144	1466	88%	0.0	96.59%	LN561701.1
Unidentified marine bacterioplankton clone P5-4B_13 16S ribosomal RNA gene, partial sequence	1144	1437	86%	0.0	96.59%	KC002238.1
Uncultured bacterium clone SHCB0981 16S ribosomal RNA gene, partial sequence	1144	1479	89%	0.0	96.59%	JN698091.1
Uncultured bacterium clone BIGO569 16S ribosomal RNA gene, partial sequence	1144	1461	88%	0.0	96.59%	HM558672.1
Uncultured bacterium clone BICP525 16S ribosomal RNA gene, partial sequence	1144	1464	88%	0.0	96.59%	HM557409.1
Uncultured bacterium clone BICP1507 16S ribosomal RNA gene, partial sequence	1144	1461	88%	0.0	96.59%	HM557341.1
Uncultured bacterium clone BICP1483 16S ribosomal RNA gene, partial sequence	1144	1466	88%	0.0	96.59%	HM557331.1
Uncultured bacterium clone BICP1350 16S ribosomal RNA gene, partial sequence	1144	1466	88%	0.0	96.59%	HM557285.1

Under microscope, the colonization capacity of DSE fungi in the roots of the rice plant was demonstrated for all species.

Veronaeopsis simplex (Y34) colonizing in the roots of 17 days old Koshihikari rice seedling which was indicated with blue-stained hyphae in Fig. 1. The colonization was indicated with resemble anastomoses structure (Fig. 1A), while the intracellular formed by *Y34* looks like microsclerotia (Fig. 1B). While the formation of blue-stained intracellular hyphae in Fig. 2A and Fig. B implied a colonization of *OGR3*.

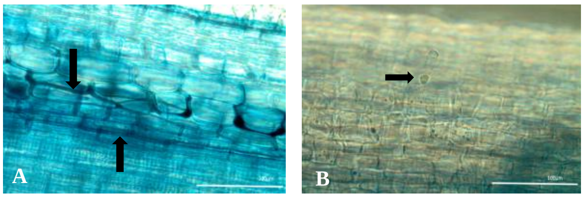

Figure 1. Colonization of *Veronaeopsis simplex (Y34)* DSE in the roots of 17 days old Koshihikari rice seedling. (A) Intercellular blue-stained hyphae forming structures resembling anastomoses; (B) melanized intracellular microsclerotia-like structure formed by (Y34).

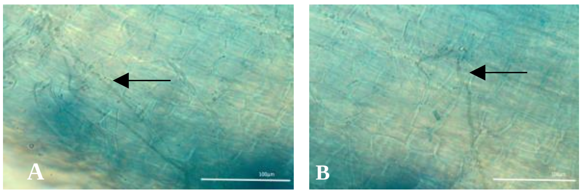

Figure 2. Colonization of *Cladophialophora chaetospira (OGR3)* DSE in the roots of 17 days old Koshihikari rice seedling. Showing formation of blue-stained intracellular hyphae (A) and (B).

For *J1PC1* DSE, the colonization demonstrated an early developmental stage of an intracellular microsclerotia (pointed with black arrow) and an early developmental stage of an intracellular microsclerotia (pointed with white arrow) (Fig. 3). Similarly, the colonization of *LtPE 2* was well shown in blue-stained intracellular hyphae (Fig. 4A), demonstrating the occurrence of melanized intracellular microsclerotia- like structure (Fig. 4B).

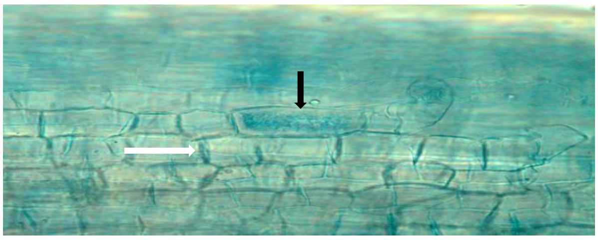

Figure 3. Colonization of *Meliniomyces variabilis (J1PC1)* DSE in the roots of 17 days old Koshihikari rice seedling showing an early developmental stage of an intracellular microsclerotia (pointed with black arrow) and an early developmental stage of an intracellular microsclerotia (pointed with white arrow).

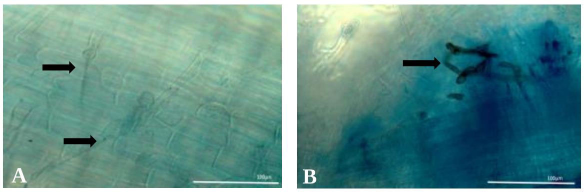

Figure 4. Colonization of *Phialocephala fortinii ($LtPE_2$)* DSE in the roots of 17 days old Koshihikari rice seedling. (A) Blue-stained intracellular hyphae; (B) occurrence of melanized intracellular microsclerotia-like structure.

The procedures of the first experiment were started on 14th November and harvested finished on 7th December 2019. Seed-borne bacteria (Table I) appeared during 24 hr after the transplantation under continuous temperature of 35 ℃ in the growth chamber. However, some characteristics of DSE fungi colonization were recognized in the roots of Koshihikari rice plant in all treatments after 17 days of transplantation. The formation of DSE fungal hyphae and micro-sclerotium (Fig. 1, Fig. 2, Fig. 3, and Fig. 4) were observed under the compound microscope with the magnification of 40X and 100X accordingly.

Previous research reported that seed endophytes can infect the next generation of the host plant through the ways or by the combination of individuals ways: (1) reside in the seed and transmitting the plant through the surfaces of other parts of the plant, and (2) remaining inside the seeds and transmit to the other parts of the plant via plant growth or move within the plant tissue, Kaga *et al.*, 2009. Truyens *et al.* 2014 reported that the internal seed endophytic bacteria which was inherited from past generation via seed and probably comprise of microbes and can tolerance desiccation and conditions of seed storage [16]. Therefore, the bacteria appeared in the first experiment can be the seed endophytic bacteria. However, further research is needed to perform to know the impact of bacteria to the host plant as well as the bacteria-plant-DSE fungi symbiosis.

B. Capacity of DSE Fungi Colonization and Root's Response to DSE Colonization under High Temperature

The root/shoot ratio of *Veronaeopsis simplex (Y34)* and *Cladophialophora chaetospira (OGR3)* treatments were highest among the four treatments (Table II). Treatment with *Cladophialophora chaetospira (OGR3)* has the highest total dry weight and treatment with *Veronaeopsis simplex (Y34)* followed second.

TABLE II. RESULT OF THE MEASUREMENTS OF THE PHYSICAL PARAMETERS OF THE PLANT GROWTH FOR THE 2ND EXPERIMENT AFTER 10 DAYS OF TRANSPLANTATION

Parameter	Control	OGR3	Y34	J_1PC_1
Shoot High (cm)	7.5	8.5	8.7	8.5
Seminal Root Length (cm)	~1.5	~2.5	~2.4	~2.2
Root Volume (cm^3)	0.1	0.1	0.1	0.1
Total Dry Weight (mg)	7.2	8.5	7.8	7.0
Shoot Dry Weight (mg)	4.1	5.4	4.9	4.4
Root Dry Weight (mg)	3.1	3.1	2.9	2.6
Root/Shoot Ratio	3:4	3:5	3:5	1:2

Notes: The results of each parameter are the mean of the five replicates for each treatment.

Under microscope, Colonization of *Veronaeopsis simplex (Y34)* DSE in the roots of 10 days old Koshihikari rice seedling was demonstrated in blue stained hyphae (Fig. 5) with melanized hyphae (white arrow) and developing ntracellular microsclerotium-like structures (black arrows).

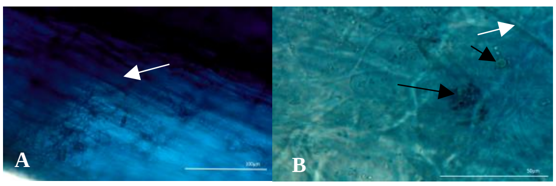

Figure 5. Colonization of *Veronaeopsis simplex (Y34)* DSE in the roots of 10 days old Koshihikari rice seedling. (A) Strong colonization of blue stained hyphae; (B) melanized hyphae (white arrow) and developing intracellular microsclerotium-like structures (black arrows).

The formation of microsclerotium-like structures (black arrows and blue-stained hyphae with white arrow are indies for the colonization of *Cladophialophora Chaetospira (OGR3)* in the plant roots (Fig. 6). Its intercellular blue-stained hyphae formed structures resembling anastomoses. While the colonization of *Meliniomyces variabilis (J1PC1)* DSE in the roots of 10 days old Koshihikari rice seedling indicated with the formation of hyphal coils pointed with black arrow (Fig. 7) and blue-stained hyphae forming structures resembling anastomoses.

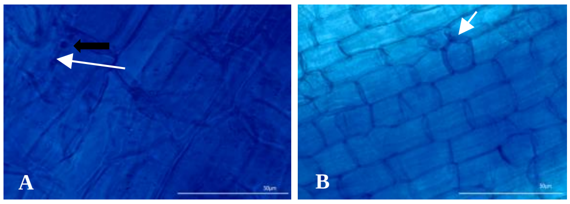

Figure 6. Colonization of *Cladophialophora Chaetospira (OGR3)* DSE in the roots of 10 days old Koshihikari rice seedling. (A) Formation of microsclerotium -like structure pointed with black arrow and blue-stained hyphae with white arrow; (B) intercellular blue-stained hyphae forming structures resembling anastomoses.

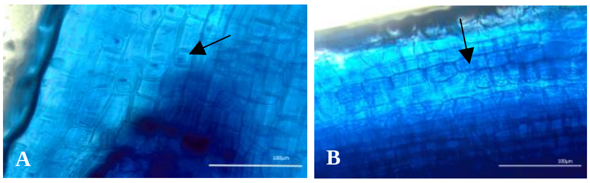

Figure 7. Colonization of *Meliniomyces variabilis (J1PC1)* DSE in the roots of 10 days old Koshihikari rice seedling. (A) Formation of hyphal coils pointed with black arrow; (B) blue-stained hyphae forming structures resembling anastomoses.

Some characteristics of DSE fungi colonization were recognized in the roots of Koshihikari rice plant in all treatments such as occurrence of DSE fungal hyphae and micro- sclerotium (Fig. 5, Fig. 6, and Fig. 7).

The second experiment were started on 24th of November and transplantation was done on the 28th of November, 2019. The harvesting was done on the 8th of December, 2019.

In this research, high temperature of 35 ℃ was used to elucidate the high-temperature impact to the symbiosis between the Koshihikari rice plant, and DSE fungi. All the selected dark septate endophytic fungi can well colonize with Koshihikari rice plant under continuous high temperature of 35 ℃ within 10 days (Fig. 5, Fig. 6, Fig. 7). Previous studies have described that some species of *Cladophialophora* can predominant at tropical and subtropical regions and produce septate, brown hyphae and unicellular conidia, meanwhile, *Veronaeopsis simplex (Y34)* DSE can be isolated from subtropical regions such as Yaku Island in Japan [9], [17]. Therefore, present study confirms that DSE fungi confer habitat-specific stress tolerance to host plant plants, Redman and Rodriguez, 2008, [18].

On the other hand, according to the Table II, measurements of the physical parameters of plant growth, treatments with DSE fungi have higher root/shoot ratio than control treatment. This result suggests that plant with DSE symbiosis can tolerate to high temperature stress than those without DSE symbiosis. Previous studies reported that fungal endophytes have different kinds of properties such as provision of nitrogen in exchange for carbon for the host plant, increase growth rate and pathogen suppression [11], [17], [19], [20]. Therefore, we can conclude that the selected DSE fungi have potential to be used as biofertilizers under high temperature condition as the adaptation tool for climate change.

IV. CONCLUSION

From the two experiments, the selected dark septate endophytes can well colonize with Koshihikari plant under a high continuous temperature of 35 ℃. Plants with DSE symbiosis have higher tolerance to high temperature stress than those without DSE symbiosis Thus, they have potential to be used as biofertilizers. However, due to the limited time, the experiment could not do for the whole life cycle of Koshihikari rice plant and the experiment need to continue to get more evidence and data.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Mai Ei Ngwe Zin conducted the research, analyzed data and co-wrote the paper. Dr. Hoang Thi Thu Duyen designed the experiment and co-wrote the paper and edited the paper. Prof. Kazuhiko Narisawa designed and supervised the research.

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