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Some Agronomical and Physiological Characteristics of Sang Yod Phatthalung Rice from *In Vitro* Regenerated Plant After Transfer to Clay Soil in Field Conditions

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Abstract

Sang Yod Phatthalung rice is indica rice and grown as Geographical Indications (GI) in Patthalung province. Its dark red color seed contains high antioxidant activity that helps reduction in the risk of some chronic diseases such as diabetes, cancer, and cardiovascular diseases. So far, yield per planting area of this rice is still low. Thus, application of PGRs might play an alternative role in agronomical and physiological characteristics leading to improving yield of this rice. First, shoot tips of Sang Yod Phatthalung rice were cultured to induce multiple shoots and root on liquified PGR-free oil palm culture medium (OPCM) with different concentrations of 6-benzyladenine (BA) (0, 0.5, 1, 1.5 mg/L) alone or in combination with 1-naphthaleneacetic acid (NAA) (0 and 0.5 mg/L). Complete plants were acclimatized to field condition at Phatthalung Rice Research Station (PRRS). After acclimatization for 120 and 140 days, some agronomical and physiological characteristics of rice plant obtained from different PGR containing OPCM were statistically compared. A 2×4 factorial design in RCBD with 4 replication was performed. The results were clearly demonstrated that adding BA in the culture medium increase plant height, number of panicles, panicle length and leaf chlorophyll content. Plantlets obtained from *in vitro* culturing shoot tip on 0.5 mg/L BA containing medium gave the highest results in average plant height at 189.50 cm, number of panicles at 34.00 panicles, panicle length at 32.50 cm and leaf chlorophyll content at 39.65 SPAD unit. The results from this present study indicated the production of new plant types or characteristics for Sang Yod Phatthalung rice for high yield in future.

Keywords: Agronomical and physiological characteristics, *In vitro,* Plant growth regulators, Regenerated plant, Sang Yod Phatthalung rice

Introduction

Rice (*Oryza sativa* L.), which belongs to the Poaceae family, is a crucial staple food and one of the most important cereal crops worldwide [1]. It is projected that there will be a 26 % increase in global demand for rice in the next 25 years, with an estimated production of nearly 555 million tons in 2035 [2].

Sang Yod Phatthalung rice, an indica rice variety, has been grown in Patthalung province for over a century and is the first cultivar in Thailand to be granted Geographical Indications (GI) status [3]. This rice is characterized by its dark-red color, which is attributed to the seed's pericarp. Compared to white rice, red rice contains higher levels of amylose (14 - 15 %), protein (8.6 per 100 g fresh weight), polyphenol (82.01 mg in gallic acid per 100 g fresh weight), and anthocyanin (15.14 mg in cyanidin-3-glucoside) [3], making it a highly nutritious food with antioxidant properties that can reduce the risk of chronic diseases such as diabetes, cancer, and cardiovascular disease [2]. As the demand for healthier rice products increases due to population growth and a desire for healthier food, producing special rice varieties for the market could increase economic profits for farmers and provide nutritional benefits to consumers. Additionally, the highly sought-after Sang Yod Phatthalung rice is expected to have high demand for future consumption [2]. However, the transformation of indica rice remains challenging due to the low frequency of plantlet regeneration, difficulties in culturing, and longer culture periods compared to japonica rice. Plant breeders can use in vitro techniques, such as mass propagation, to create new species and characteristics that differ from the wild type, potentially increasing certain chemical compositions and antioxidant properties [4]. Successful application of these techniques requires appropriate plantlet regeneration methods.

Numerous authors have reported the use of plant growth regulators (PGRs) in rice tissue culture. In our previous study, it was found that the oil palm culture medium (OPCM) was the most effective medium

for propagating the Sangyod rice cultivar [5]. PGRs are extensively used in culture medium to enhance plantlet regeneration and improvement pathway of plant cell development. Cytokinin was used to stimulate cell division and promote growth and development of axially and adventitious shoots. Previous studies have reported that the combination of cytokinin and auxin promotes plantlet regeneration in rice [2,6].

Previous study reported the positive effect of BA on agronomical results of cultivated cereals. Gupta *et al.* [7], and Zheng *et al.* [8], reported in *Triticum aestivum* L. that application of BA could increase grain weight. Hosseini *et al.* [9], reported in *Hordeum vulgare* L. that spraying plants with a synthetic cytokinin could improve yield. Moreover, reporting in *Oryza sativa* L. cv. Hom Kra Dang Nga rice [10], and Sang Yod Phatthalung rice [11], that adding BA and NAA could increase number of shoots, leaves and roots, leaf and root length.

For physiological result, Gao *et al.* [12], reported in *Zea mays* L. that adding BA improve photosynthesis and accelerate crops grain productivity. In flowering plant, Cioc *et al.* [13], reported that adding BA in the medium for culture *Gerbera jamesonii* resulted in higher total chlorophyll contents in the leaves. BA is cytokinin that plays an important role in the development and structural differentiation of chloroplasts and also activate genes involved in their development in *in vitro* cultures like those reported by Dobranszki and Mendler-Drienyovszki [14]. Moreover, this cytokinin inhibited leaf senescence due to hamper chlorophyll break down, control plant morphometry and photosynthetic pigment content. However, application at high concentration may cause a reduction of those phenomena. Results were also reported by Sardoei *et al.* [15], in indoor ornamental plants that application of BA in higher concentrations had positive effects on leaf chlorophyll content of *Ficus benjamina*, *Schefflera arboricola* and *Dizigotheeca elegantissima* foliage plants. In addition, it is well known that different optimal cytokinin concentrations exist for different physiological processes in plants [16]. Obviously, in excised zucchini cotyledons the concentration of 45 μM BA turned to be supra optimal for chlorophyll synthesis when BA was applied together with light [17], indicating the maximum photosynthesis. However, to author knowledge, there have been no reports on the combined effect of cytokinin and auxin on plantlet regeneration under field conditions, nor on the agronomic characteristics of the Sang Yod Phatthalung rice cultivar following such treatment.

The aim of this study was to determine the optimal concentrations of BA or NAA for inducing multiple shoot formation through shoot tip culture, as well as to evaluate the agronomic and physiology characteristics of the resulting plants following acclimatization in field conditions.

Materials and methods

Plant material

In this experiment, the first putative somaclonal variants from the first regeneration (SCV1R1) of Sang Yod Phatthalung rice seeds were used. These seeds were obtained from multiple shoot culturing in PGR-free OPCM, as well as OPCM supplemented with different types and concentrations of PGRs (0.5 mg/L BA, 1 mg/L BA with 0.5 mg/L NAA, and 1.5 mg/L BA with 0.5 mg/L NAA), as previously reported. The resulting seeds were then germinated to produce growing plant materials, which were classified into 4 groups as follows.

Group I: Ex vitro grown plants

Mature seeds of SCV1R1 were germinated on tissue paper and sprayed with water every day for 7 days. Germinated seedlings were transferred to field conditions at Phatthalung Rice Research Center (PRRC). Seedlings obtained from this group were called *Ex*SCV1R1 and divided into 4 subgroups as following:

1.1: *Ex*SCV1R1 from PGR- free OPCM (ExSCV1R1-PGR)
1.2: *Ex*SCV1R1 from 0.5 mg/L BA (ExSCV1R1+0.5BA)
1.3: *Ex*SCV1R1from 1 mg/L BA with 0.5 mg/L NAA (ExSCV1R1+1BA0.5NAA)
1.4: *Ex*SCV1R1 from 1.5 mg/L BA with 0.5 mg/L NAA (ExSCV1R1+1.5BA0.5NAA)

Group II: In vitro grown plants

In this group, mature seeds of SCV1R1 were dehusked and washed with running tap water for 20 min, surface sterilized with 70 % ethanol for 2 min and immersed in 20 % Clorox with 3 drops Tween 20 for 6 min. Finally, the seeds were rinsed with sterile distilled water for 3 times in a laminar air flow hood and blotted on sterile tissue paper. Disinfected seeds were cultured on OPCM medium without PGRs, and supplemented with 3 % (w/v) sucrose and solidified with 0.7 % (w/v) agar. The pH of the culture medium was adjusted to 5.7 before autoclaving at 121 °C, 1.07 kg/cm^2 for 20 min. All cultures were carried out in Petri-plate (Ø 9 cm), sealed by Parafilm and maintained at 26 ± 2 °C in the culture room under 10 h

photoperiod with an irradiance of 25 μmol/m^2/s provided by cool white fluorescent tubes. After culture for 30 days, seedlings were obtained and shoot tip explants were used for shoot proliferation.

A single shoot was excised and cut the leaves above the growing point to retain the shoot explant at a size of 5 mm in length, then, transferred to culture in liquid OPCM medium with 0 - 1.5 mg/L BA or 0.5 mg/L NAA to induce multiple shoots and root. The cultures were maintained on a rotary shaker at a speed of 100 rpm for 3 weeks. Complete plantlets (shoot with root) were acclimatized and grown at PRRS.

Thus, plant materials obtained from this group were called *In*SCV1R2 plants and divided into 4 subgroups as follows:

2.1: *In*SCV1R2 from SCV1R1 seeds in PGR-free OPCM (*In*SCV1R2-PGR)

2.2: *In*SCV1R2 from SCV1R1 seeds in OPCM with 0.5 mg/L BA (*In*SCV1R2+0.5BA)

2.3: *In*SCV1R2 from SCV1R1 seeds in OPCM with 1 mg/L BA and 0.5 mg/L NAA (*In*SCV1R2+1BA0.5NAA)

2.4: *In*SCV1R2 from SCV1R1 seeds in OPCM with 1.5 mg/L BA and 0.5 mg/L NAA (*In*SCV1R2+1.5BA0.5NAA)

Group III: Ex vitro original seed (ExOS; control treatment)

Ex vitro original mature seeds of Sang Yod Phatthalung rice kindly provided by PRRC at Phatthalung province were used as compared treatment. The seeds were germinated and grown under the same procedures as group I.

Group IV: In vitro original seed (InOS; control treatment)

In vitro original mature seeds of Sang Yod Phatthalung rice kindly provided by PRRC at Phatthalung province were used as compared treatment. The seeds were germinated and grown under the same procedures as group II.

Effect of culture condition and PGRs from previous culture medium on agronomical and physiological characteristics

In this investigation, a randomized complete block design (RCBD) in factorial was performed. Factor A was culture condition which was 2 conditions, *in vitro* and *ex vitro* one. Factor B was concentrations of PGRs in previous culture medium which was 4 different concentrations (according to 1.1 - 1.4 and 2.1 - 2.4). Both *in vitro* and *ex vitro* plants or seedlings were transferred to 12-inch plastic pot containing clay soil and maintained under the natural light condition at 28 - 30 °C in Phattalung Rice Research Station (PRRS). Water was filled to full pot every 2 days, and 0.1 g/pot fertilizer (16-20-0) was applied once a month. For pest management fenpyroximate was used disinfestation red mite, and hexaconazole disinfestated brown spot disease, spray 10 cc of insecticide per 20 L of water. After raising under those conditions for certain periods some agronomical characteristics were recorded and compared statistically among those 2 different culture conditions and 4 concentrations of PGRs in the previous culture medium which was 4 different concentrations (according to 1.1 - 1.4 and 2.1 - 2.4). Plant height was measured from culm at ground level to the top or new emerging leaf at 120 days after raising. After flowering and fruit set, the number of panicles and panicle length were recorded at 140 days after raising. For physiological characteristic, chlorophyll content SPAD was measured at the 2nd leaf from the top by digital chlorophyll meter SPAD 502 plus and the data were recorded at 120 days after raising.

Each treatment combination was analyzed using 2×4 factorial design in Randomized Complete Block Design (RCBD) with 4 replicates per treatment. Means among each factor and treatment combination were separated by Fisher's Least Significant Difference (LSD) ($p \leq 0.05$) using the program R statistical package version 2.14.2.

Results and discussion

Effect of culture condition and PGRs from previous culture medium on agronomical and physiological characteristics

In the present study, the effect of concentrations of BA with NAA in previous culture medium before transferring plant to soil on agronomical characteristics were compared.

For plant height the result showed that after 120 days of transfer, plantlets from 0.5 mg/L BA-derived plants germinated *ex vitro* gave the highest result at 189.50 cm, significantly different ($p < 0.05$) with that obtained from other treatments. Moreover, PGRs and culture conditions affect plant height. There was an interaction between PGRs and culture conditions. (**Figures 1** and **2**).

For the number of panicles, the result showed that after 140 days of transfer, plantlets from 0.5 mg/L BA -derived plants germinated *in vitro* gave the highest result at 34.00 panicles, not significantly different ($p < 0.05$) with PGR-free, 1 mg/L BA + 0.5 mg/L NAA, original seeds-derived plants germinated culture condition. Moreover, PGRs and culture conditions affect the number of panicles. There was an interaction between PGRs and culture conditions (**Figures 3** and **4**). For panicles length the results showed that after 140 days of transfer, plantlet from 0.5 mg/L BA derived plants germinated *in vitro* gave the highest result at 32.50 cm, significantly different ($p < 0.05$) with 1.5 mg/L BA + 0.5 mg/L NAA derived plants germinated *in-* and *ex vitro* and original seeds derived plants germinated *ex vitro*. Moreover, PGRs affect panicles length. There was interaction between PGRs and culture conditions. However, the condition non affects panicles length (**Figures 4** and **5**).

The present studies, compared the effect of concentrations of BA with NAA in the previous culture medium before transferring plant to the soil on physiological characteristics.

For leaf chlorophyll content the results showed that after 120 days of transfer, plantlet from 0.5 mg/L BA *in vitro* raised plants gave the highest result at 39.65 SPAD unit, significantly different ($p < 0.05$) with 1.5 mg/L BA + 0.5 mg/L NAA and original seeds derived plants germinated *ex vitro* and *in vitro*. Moreover, PGRs or culture conditions affect plant height. There was not interaction between PGRs and culture condition (**Figures 6** and **7**).

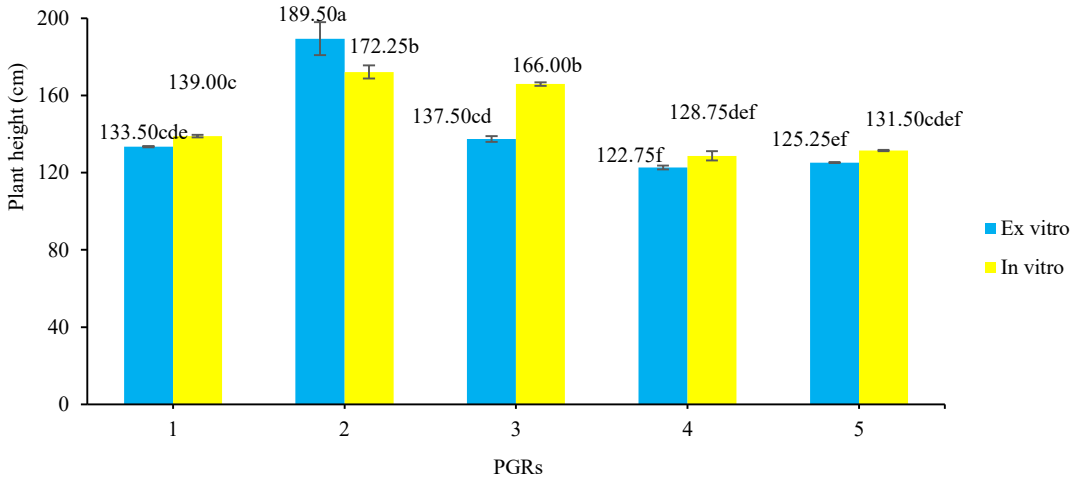

Figure 1 Effects of BA and NAA containing liquified OPCM medium on plant height from a single shoot of Sang Yod Phatthalung rice after transfer to clay soil for 120 days.

Means showing different letters between histogram are significantly different by LSD
1 = PGR-free
2 = 0.5 mg/L BA
3 = 1 mg/L BA + 0.5 mg/L NAA
4 = 1.5 mg/L BA + 0.5 mg/L NAA
5 = original seeds
Mean sharing different small letters show significantly different among treatment combinations LSD, significant different ($p < 0.05$), C.V. = 8.66 %

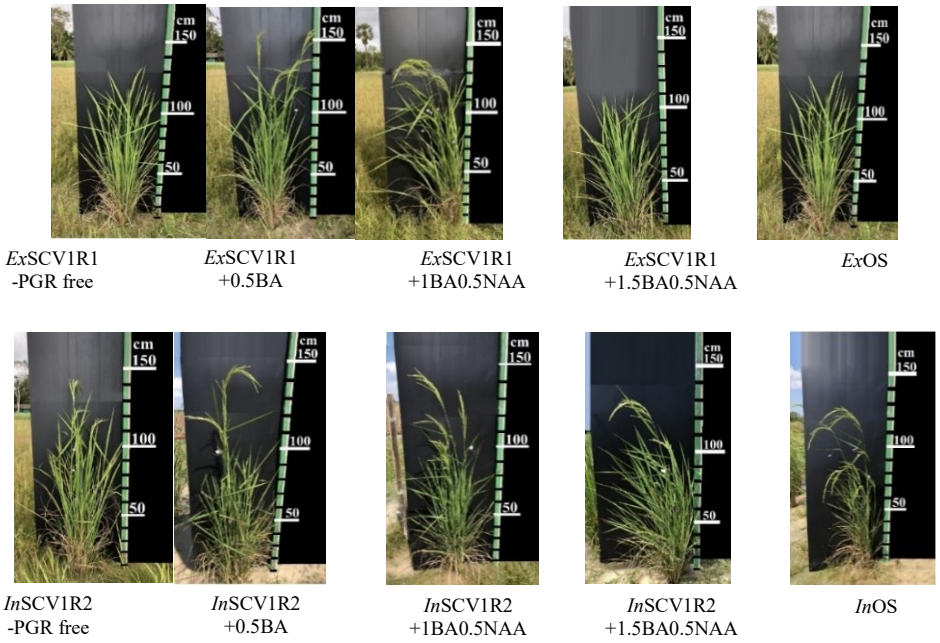

Figure 2 Agronomical characteristics of plant of Sang Yod Phatthalung rice after transfer to clay soil until flowering in the field conditions of PRRC for 120 days.

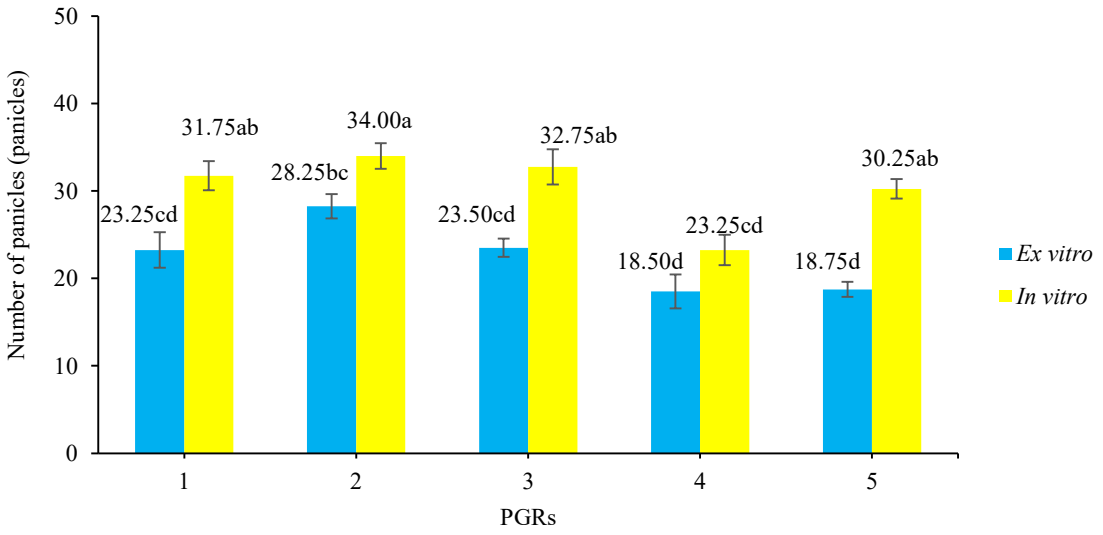

Figure 3 Effects of BA and NAA containing liquified OPCM medium on the number of panicles from a single shoot of Sang Yod Phatthalung rice after transfer to clay soil for 140 days.

Means showing different letters between histogram are significantly different by LSD
1 = PGR-free
2 = 0.5 mg/L BA
3 = 1 mg/L BA + 0.5 mg/L NAA
4 = 1.5 mg/L BA + 0.5 mg/L NAA
5 = original seeds
Mean sharing different small letters show significantly different among treatment combinations LSD, significant different ($p < 0.05$), C.V. = 2.00 %

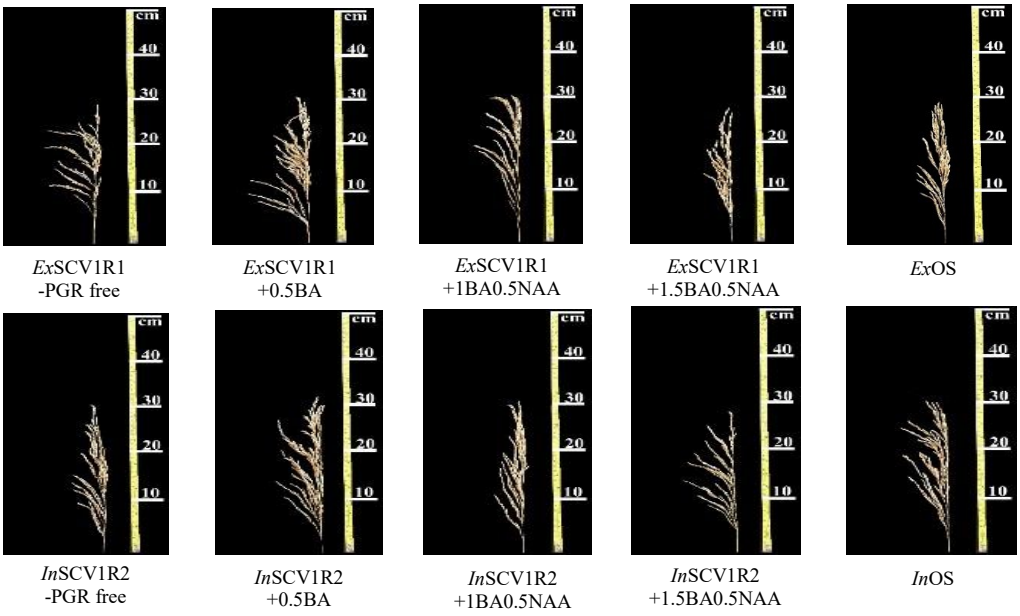

Figure 4 Characteristics of panicles of Sang Yod Phatthalung rice harvested from field grown plants at PRRS.

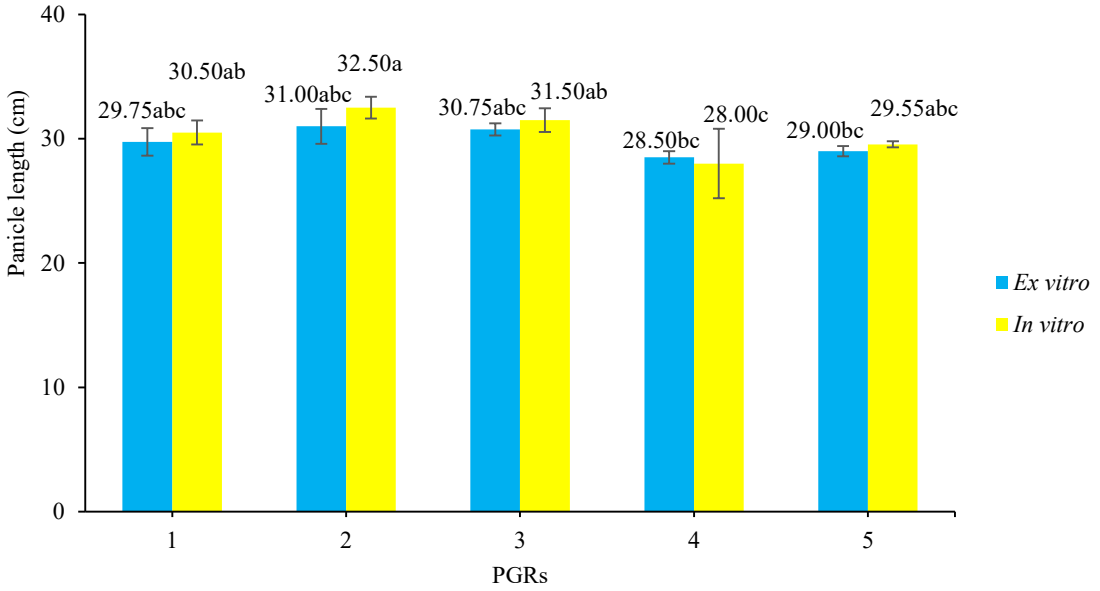

Figure 5 Effects of BA and NAA containing liquified OPCM medium on panicle length from a single shoot of Sang Yod Phatthalung rice after transfer to clay soil for 140 days.

Means showing different letters between histogram are significantly different by LSD
1 = PGR-free
2 = 0.5 mg/L BA
3 = 1 mg/L BA + 0.5 mg/L NAA
4 = 1.5 mg/L BA + 0.5 mg/L NAA
5 = original seeds

Mean sharing different small letters show significantly different among treatment combinations LSD, significant different ($p < 0.05$), C.V.= 8.66 %

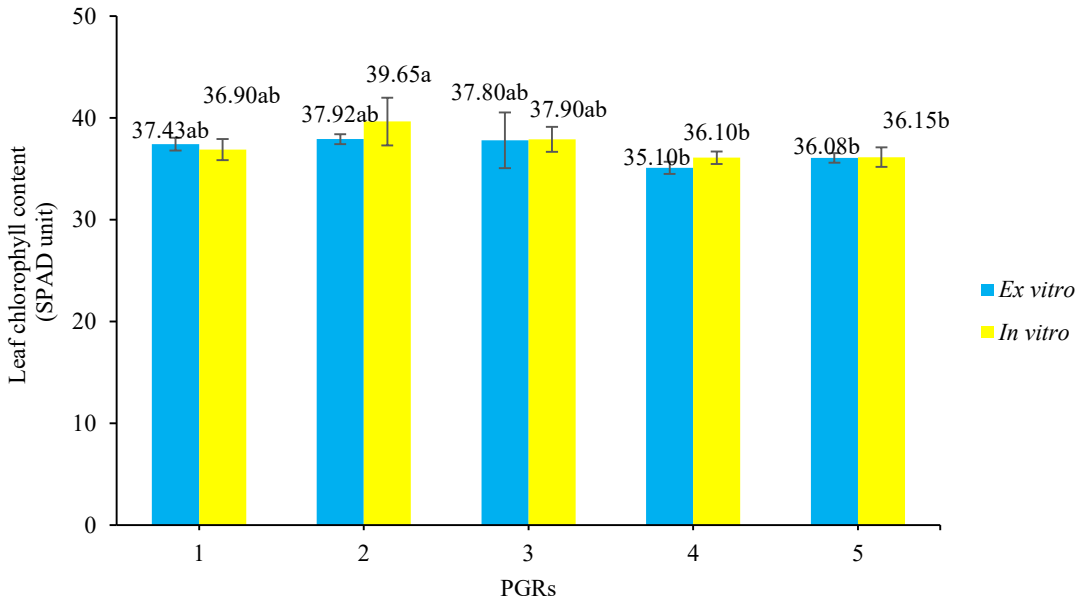

Figure 6 Effects of BA and NAA containing liquified OPCM medium on leaf chlorophyll content from a single shoot of Sang Yod Phatthalung rice after transfer to clay soil for 80 days.

Means showing different letters between histogram are significantly different by LSD
1 = PGR-free
2 = 0.5 mg/L BA
3 = 1 mg/L BA + 0.5 mg/L NAA
4 = 1.5 mg/L BA + 0.5 mg/L NAA
5 = original seeds
Mean sharing different small letters show significantly different among treatment combinations LSD, significant different ($p < 0.05$), C.V. = 9.04 %

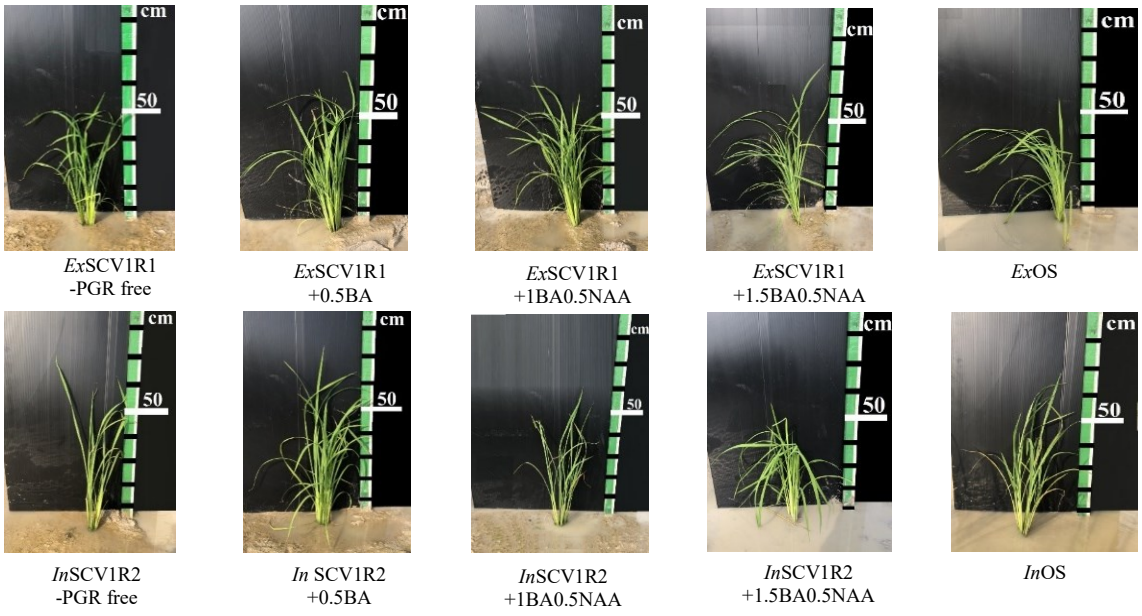

Figure 7 Agronomical characteristics of Sang Yod Phatthalung rice after transfer to clay soil in the field conditions of PRRC for 80 days.

In this study, concentrations of BA and NAA used in the previous culture medium played a vital role in agronomical and physiological characteristics. To date, there have been no reports on the growth and yield comparison between *in vitro*-propagated rice plants and conventionally seed-derived plants. Author study is pioneering in demonstrating that *in vitro*-propagated rice plants have superior growth and yield compared to conventionally seed-derived plants. Additionally, leaves from *in vitro*-propagated rice had higher chlorophyll content, likely due to the use of a high concentration of BA in the culture medium that enhanced the formation of chlorophyll. These findings suggest that in vitro propagation techniques may have potential applications for improving the growth and yield of rice crops. A similar result was also reported in *Ficus benjamina*, *Schefflera arboricola* and *Dizigotheeca elegantissima* flower that application of BA alone or in combination with GA_3 promoted the increment of chlorophyll at every time of recording [15]. BA alone in a culture medium (Murashige and Skoog medium) could enhance the increment of chlorophyll content. Plant growth regulators, especially BA and kinetin (KN) are a component of cytokinin, promote the synthesis of chlorophyll and the development of chloroplast [18]. These results were in accordance with Liu *et al.* [19], who explained that the application of gibberellic acid increased chlorophyll content in chamomile. Similarly, Singh and Hippalgaonkar [20], explained the application of kinetin increased chlorophyll content. However, BA alone was used in the current study; GA_3, KN, and gibberellic acid were not. Nevertheless, in the future, interactions involving BA, GA_3, KN, and gibberellic acid might be employed. In addition, Yan *et al.* [21], reported that cytokinin are involved in control biogenesis and function of chloroplast such as rate of photosynthesis, pigment accumulation and chloroplast enzyme activity. Exogenously applied cytokinin keep chloroplast photosynthetically active longer than leaves not treated with cytokinin [22]. Cytokinin result from hormone effects on expression of nuclear genes encoding chloroplast proteins [23]. This hormone affected chlorophyll synthesis because cytokinin signaling pathway involving in transduction cytokinin signal to transcription factors regulating chloroplast development and function. These comprise cytokinin receptors AHK2, AHK3, ARR1, ARR10, ARR12, and CRF2, which send signals to receptors GNC, CGA1, and PDV2. GNC and CGA1 regulate several aspects of chloroplast development and plastid division. Furthermore, CRF2 increases level of PDV2, which is required for plastid division [24]. In summary, adding BA (cytokinin) to the medium causes the chlorophyll content to increase, resulting in the plant height, panicle number, and length to increase as well. This may explain the differences between the *in vitro*-propagated and the seed-grown plants in the plant height, number of panicles, panicles length and leaf chlorophyll content under field conditions.

During *in vitro* culture period, BA was added to the culture medium (OPCM). A carry-over effect of this hormone under the field conditions, which facilitated the formation of more tillers and higher stem in the *in vitro*-propagated rice, is likely Shima banana [25-27]. For planting of *in vitro*-propagated banana (from BA containing Murashige and Skoog (MS) medium) in the field conditions it was reported that they produced a number of suckers nearly 2 times higher than those of conventional banana (sucker-derived banana) [10]. Moreover, Sistichan *et al.* [28], reported that the application of BA to pineapple stem cutting by prolonged soaking method could promote axillary shoot growth and significantly increase the number of new shoots compared to control (0 ppm BA). Similar result in *Phaseolus vulgaris* L. treated with 30 mg/L BA [29], *Andropogon gerardi* Vitman treated with 5 mg/L BA [30], *Solanum melongena* L. treated with 2.25 mg/L BA [18], *Ficus benjamina, Schefflera arboricola and Dizigotheeca elegantissima* treated with 200 mg/L BA [15], summer maize treated with 100 mg/L BA [31], *Landoltia punctata* treated with 0.23 mg/L BA [19], affect high chlorophyll content. BA increase chlorophyll content due to BA (cytokinin) linked to chloroplast behavior. Molecular mechanisms of cytokinin action on chloroplast division and development are well documented [24].

Conclusions

Sang Yod Phatthalung is a plant species that exhibited varied responses after being transferred from *in vitro* conditions to grow in a PRRC (planting room with soil) environment. The preparation of the plant material before transfer was found to have a significant impact on both the agronomical and physiological characteristics of the plants. Among the different treatments tested, the use of 0.5 mg/L BA in the *in vitro* technique provided the best results in terms of agronomical (number of panicles and panicles length) and physiological characteristics (leaf chlorophyll content). These findings suggest that careful preparation of plant material using appropriate techniques can improve the growth and development of plants, and may have implications for the cultivation of other plant species as well.

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