INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–967/2015/17–5–1049–1054
DOI: 10.17957/IJAB/15.0030
http://www.fspublishers.org

Full Length Article

Establishment of Condition and Nanoparticle Factors Influencing Plant Regeneration from Aromatic Rice (*Oryza sativa*)

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Abstract

Improvements on the callus induction and regeneration stages as well as the applications of nanoparticles using aromatic rice cv. KDML105 were investigated in this study. An efficient induction of plant regeneration from mature seeds was found in calli cultured under dark condition and was not transferred to the fresh medium (non-subculture) in each culture stage. Although, the size and biomass factors showed the better performance in calli cultured under light condition than dark but the regeneration frequency rate was lower than dark condition. Between the types and concentrations of nanoparticles applied for the regeneration enhancement. ZnO nanoparticles were found to be toxic, while TiO_2 nanoparticles improved the plant regeneration. Optimum concentration (25 mg L^{-1}) of TiO_2 nanoparticles showed the high rate of green spots, plant regeneration and the ratio of the number of seedlings to the number of regenerated calli. Nonetheless, these factors may be valuable to establish an improvement in plant tissue culture system for propagation and/or genetic transformation in aromatic rice.

Keywords: Aromatic rice; Callus induction; Nanoparticles; Plant regeneration

Introduction

Now-a-days, crop productions are affected from biotic and abiotic stresses and becoming a big problem worldwide. This problem is causing the reduction in crop production, including rice yields (Geng *et al.,* 2008). Moreover, rice consumers are increasing in every day whereas the rice cultivation areas are decreasing (Karthikeyan *et al.,* 2012). *In vitro* culture system or tissue culture technique is a key step for the crop improvement of the rice cultivars that collaborate with genetic transformation and traditional breeding (Lee *et al.,* 2002; Dabul *et al.,* 2009; Muhammad et al., 2014). The adaptation protocol requires high frequency in callus induction and subsequent plant regeneration. Success in the enhancement of callus induction and plant regeneration depends on the various factors including plant cultivar, media composition and culture condition (Hoque and Mansfield, 2004). *Indica* rice is widely produced and consumed in the world more than the *japonica* types. Many researchers have reported the tissue culture manipulation of *indica* rice cultivar, which is still a challenging due to low frequency of callus induction and plant regeneration abilities, especially aromatic rice (Khaleda and Al-Forkan, 2006; Kumria *et al.,* 2000; Zuraida *et al.,* 2010).

In recent years, the nanoparticles are used in many field researches and have been commonly used as electronic, textiles, optical filters, catalysts and sensors (Carmen *et al.,* 2003; Viana *et al.,* 2010). The many studies involved the nanoparticle applications in agricultural research, such as toxicity and productivity in plant, animal and microorganism (Castiglione *et al.,* 2011; Griffitt *et al.,* 2007; Jones *et al.,* 2008). The results have presented both negative and positive effects of the different types and concentrations of nanoparticles (Lu *et al.,* 2002; Yang and Watts, 2005; Navarro *et al.,* 2008). A few researches have studied the effect of nanoparticles on plant cell culture and/or plant regeneration. This research established the enhancement of plant regeneration in aromatic rice (KDML105 cultivar) using the different condition of the callus induction and plant regeneration stages. Moreover, it was also investigated the effect of nanoparticles (ZnO and TiO_2) on the plant regeneration frequency for the study of plant line improvement and genetic transformation in the future.

Materials and Methods

Plant Materials

The seeds of aromatic *indica* rice cultivar (*Oryza sativa* L. cv. Khao Dawk Mali 105; KDML105) were used for the callus induction and plant regeneration. The dehusked seeds were sterilized by 70% ethanol for 2–3 min, followed by 5% commercial bleach (5.25% sodium hyprochloride) for 30 min and 30% commercial bleach for 30 min. The seeds were thoroughly rinsed 4–5 times with sterilized distilled water. Then, the seeds were dried on sterilized tissue paper.

To cite this paper: Chutipaijit, S., 2015. Establishment of condition and nanoparticle factors influencing plant regeneration from aromatic rice (*Oryza sativa*). *Int. J. Agric. Biol.*, 17: 1049–1054

Callus Induction and Plant Regeneration

Surface-sterilized seeds were aseptically cultured on NB medium (Li *et al.,* 1993) supplemented with 2 mg L^{-1} 2,4-D (dichlorophenoxyacetic acid), 500 mg L^{-1} L-proline, 500 mg L^{-1} L-glutamine, 300 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose and 8 g L^{-1} agar with pH 5.6–5.8. The experiments were tested from various culture conditions as described below.

i) To study the effect of light/dark conditions on the callus induction and plant regeneration. The cultures were either incubated at 25 ±2ºC in different light conditions, dark condition and 16/8 h photoperiod (1000 lux) condition. The percentages of callus induction were calculated using the ratio of number of induced calli to the number of cultured seeds. After 3 weeks, calli were excised and desiccated on sterilized tissue paper for 1 week and incubated at the same condition. Then, the desiccated calli were transferred to regeneration medium [NB medium supplemented with 5 mg L^{-1} BA (benzyl aminopurine), 1 mg L^{-1} IAA (indoleacetic acid), 500 mg L^{-1} L-proline, 300 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose and 5 g L^{-1} Phytagel® with pH 5.6–5.8 and incubated at 25 ±2ºC under 16/8 h photoperiod (1000 lux). After 4 weeks, the number of green spots, shoot buds and ratio of the number of seedlings to the number of regenerated calli were recorded.

ii) To study the effect of culture condition on the callus induction and plant regeneration stages. The cultures were either incubated at 25 ±2ºC in dark condition or 16/8 h photoperiod (1000 lux). After 1 week, calli were transferred to fresh culture medium of the same composition and incubated under the same light condition. After 2 weeks, calli were excised, desiccated and transferred to regeneration medium in the same medium and condition of described in section (i). After 1 week of the regeneration stage, calli were either transferred to fresh culture medium (regeneration medium) of the same composition or continually incubated under the same condition. The schematic of culture condition is illustrated in Fig. 1.

iii) To study the effect of nanoparticles on the plant regeneration, the appropriate conditions from section (i) and (ii) were used. The zinc oxide (ZnO; average size 30.40 ±3.10 nm; Global Chemical Co., Ltd., Thailand) and titanium dioxide (TiO_2; average size 24.00 ±1.90 nm; Evonik Degussa GmbH, Germany) nanoparticles were added on the regeneration medium with concentrations of 0, 5, 25 and 50 mg L^{-1} at regeneration stage.

Statistical Analysis

The experiment was arranged in completely randomized design (CRD) with three replicates (n=3). The mean values were compared by *t*-test or Duncan's New Multiple Range Test (DMRT) and analyzed by SPSS software (SPSS for Windows version 15, SPSS Inc., Chicago, USA).

Results

The Effect of Light/dark Conditions on the Callus Induction and Plant Regeneration

The sterilized seeds were either incubated in dark condition or 16/8 h photoperiod for 3 weeks to estimate the effect of light on the callus induction. After 1 week, the small calli were induced from the scutellum region of mature seeds. The calli cultured in dark condition were showed dry and compact characters, whereas the calli cultured under light condition showed wet and friable characters (Fig 2). The percentages of callus induction were insignificant difference of calli among dark and light conditions (Table 1). After 3 weeks, the size, fresh weight (FW) and dry weight (DW) of calli incubated in light condition (2.07 cm, 304.72 mg and 34.00 mg, respectively) were higher than the calli incubated in dark condition (0.97 cm, 102.07 mg and 10.73 mg, respectively) (Table 1). And then, the desiccated calli of both conditions were transferred to the regeneration medium. Green spots were appeared from the callus surface after cultured on the regeneration medium for 1 week. After 2 to 4 weeks, green spots were differentiated into shoot bud formations. On the contrary, the calli incubated in dark condition (the callus induction stage) that transferred to regeneration medium showed 95.86% green spots, 56.26% regeneration frequency and 2.58, the ratio of the number of seedlings to the number of regenerated calli which more than the calli incubated in light condition (the callus induction stage) (Table 2).

The Effect of Culture Condition on the Callus Induction and Plant Regeneration Stages

The calli were transferred to fresh culture medium of the same composition after 1 week incubation in callus induction and/or regeneration stages to assess the effect of subculture condition on the callus induction and regeneration frequency. Following subculture after 1 week, pale-yellow to brown callus appeared on the surface of callus (Fig. 2). The highest rate of green spots (95.86%), regeneration frequency (55.25%) and the ratio of the number of seedlings to the number of regenerated calli (2.58) was found from the calli induced at dark and non-subculture conditions in the callus induction and regeneration stages (Treatment D) (Table 3). On the contrary, the calli induced in light and subculture conditions in the callus induction and regeneration stages (Treatment E) showed the lowest rate of green spots, regeneration frequency and the ratio of the number of seedlings to the number of regenerated calli, respectively. According to the results, the subculture condition had the low potential for plant regeneration.

Table 1: The effect of dark and light conditions on the percentages of callus induction, size, fresh weight (FW) and dry weight (DW) from the seeds of aromatic rice cv. KDML105 cultured on the callus induction stage

Conditions	% Callus induction	Size (cm)	FW (mg)	DW (mg)
Dark	91.32 ±0.82 a	0.97 ±0.03 b	102.07 ±1.87 b	10.73 ±0.13 b
Light	91.12 ±1.11 a	2.07 ±0.06 a	304.72 ±0.98 a	34.00 ±0.58 a

Mean values were taken from average of three replication (n=3). Mean values ±SD followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to *t*-test

Table 2: The effect of dark and light conditions on the percentages of green spots, regeneration frequency and the ratio of the number of seedlings to the number of regenerated calli from the calli of aromatic rice cv. KDML105 cultured on the regeneration stage

Conditions	% Green spots	% Regeneration frequency	The ratio of seedlings/regenerated calli
Dark	95.86 ±1.67 a	55.25 ±1.22 a	2.58 ±0.10 a
Light	94.65 ±1.76 b	54.85 ±1.69 b	2.38 ±0.04 a

Mean values were taken from average of three replication (n=3). Mean values ±SD followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to *t*-test

Table 3: The effect of culture conditions at the callus induction and/or regeneration stages on the percentages of green spots, regeneration frequency and the ratio of the number of seedlings to the number of regenerated calli from the calli of aromatic rice cv. KDML105 cultured on the regeneration stage

Treatments	Conditions	Callus induction stage	Regeneration stage	% Green spots	% Regeneration frequency	The ratio of seedlings/regenerated calli
A	Dark	+	+	68.79 ±1.84 e	25.30 ±1.24 f	1.48 ±0.09d
B	Dark	+	-	79.19 ±0.70 c	38.59 ±1.95 c	1.73 ±0.03b
C	Dark	-	+	72.93 ±0.35 d	29.19 ±1.67 d	1.64 ±0.04c
D	Dark	-	-	95.86 ±1.67 a	55.25 ±1.22 a	2.58 ±0.10a
E	Light	+	+	67.68 ±1.75 e	24.75 ±1.72 f	1.52 ±0.04d
F	Light	+	-	78.48 ±1.67 c	38.69 ±1.76 c	1.67 ±0.03c
G	Light	-	+	73.13 ±0.35 d	26.87 ±0.35 e	1.56 ±0.06cd
H	Light	-	-	94.65 ±1.76 b	54.85 ±1.69 b	2.38 ±0.04a

Mean values were taken from average of three replication (n=3). Mean values ±SD followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Duncan's New Multiple Range Test (DMRT)

"+" = After 1 week of the incubation, the calli were transferred to fresh culture medium of the same composition at this stage

"-" = After 1 week of the incubation, the calli were not transferred to fresh culture medium of the same composition at this stage

The Effect of Nano-particles on the Plant Regeneration

The dark and non-subculture condition in the callus induction and regeneration stages (Treatment D) were used for this study. Among the different concentrations of ZnO nanoparticles, the percentages of green spots and regeneration frequency from the calli cultured on the regeneration medium supplemented with 5, 25 and 50 mg L^{-1} were decreased when compared to the calli cultured on the regeneration medium without ZnO nanoparticles and showed insignificant difference of the ratio of the number of seedlings to the number of regenerated calli (Table 4). While, the addition of 25 mg L^{-1} TiO_2 nanoparticles to the regeneration medium increased the percentages of green spots (94.96%), regeneration frequency (56.46%) and the ratio of the number of seedlings to the number of regenerated calli (2.80) when compared to the other treatments (Table 5; Fig. 3). This result indicated that ZnO nanoparticles had toxic effects on plant regeneration whereas the proper concentration of TiO_2 nanoparticles enhanced the regeneration frequency in aromatic rice cultivar.

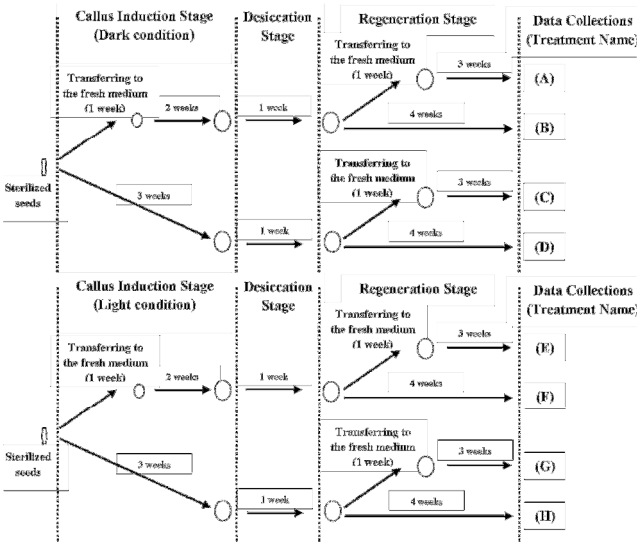

Fig. 1: Experimental procedure for the study in effect of the subcultured condition on the callus induction and plant regeneration frequency

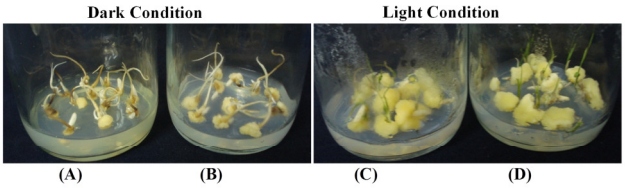

Fig. 2: Characterizations of calli from mature seeds in aromatic rice cv. KDML105 after 3 weeks of callus initiation. (A) calli were subcultured on the fresh medium after 1 week and (B) calli were non-subcultured on the fresh medium after 1 week under dark condition; (C) calli were subcultured on the fresh medium after 1 week and (D) calli were non-subcultured on the fresh medium after 1 week under light condition

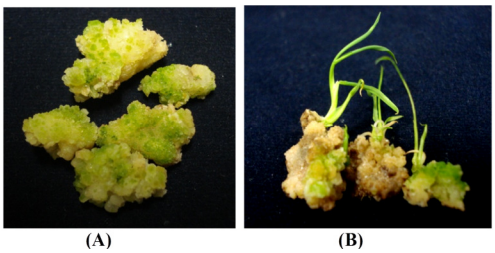

Fig. 3: Characterizations of green spots (A) and regenerated plantlets with shoots (B) in the regeneration medium supplemented with 25 mg L^{-1} TiO_2 nanoparticles

Discussion

The efficiency of callus induction and plant regeneration properties are known to depend on the culture condition and components (Chauhan and Kothari, 2004; Blando *et al.,* 2013; Ali et al., 2014). The effects of light and dark conditions on the callus induction were studied in this study. A few researches are available on comparison of light and dark conditions in callus induction stage from rice seeds and hence, an interesting factor for studies on regeneration improvement from *indica* rice varieties. The results showed that calli cultured under light condition gave better size and biomass than calli cultured under dark condition and was no significant difference in the percentages of callus induction. The previous researches have been reported in accordance with these results (Liu *et al.,* 2001; Kamal *et al.,* 2009; Afrasiab and Jafar, 2011). On the other hand, plant regeneration frequency of calli from dark condition showed higher performance than calli from light condition when cultured on regeneration medium. Although light irradiation during callus induction stage was required for biomass enhancement but plant regeneration frequency was not improved in aromatic rice cv. KDML105. The mature seeds cultured under dark condition may induce the embryogenic callus more than the mature seeds cultured under dark condition which found predominant regeneration frequency.

In this study, the culture components not only found to affect but the culture conditions had also impact on the efficiency of plant regeneration. The subculture medium was influenced in the callus morphology potential (Zuraida *et al.,* 2011). The results showed that the non-subculture condition during the callus induction and/or regeneration stage (Treatment D, Table 3) was appropriate for regeneration enhancement. The subcultured callus turned brown during the callus induction and regeneration stages, so these conditions were inappropriate for callus culture. The subcultured calli were non-embryogenic when it produced less green spots and shoot buds after transferred to the regeneration medium (Zuraida *et al.,* 2011). Unlike the previous studies, calli were proliferated and activated for the high efficiency in plant regeneration when transferred to fresh medium (Pons *et al.,* 2000; Hoque and Mansfield, 2004; Geng *et al.,* 2008; Wani *et al.,* 2011). The subculture condition may be promoting the plant regeneration for the specific species/cultivar but the subculture condition was unsuitable and necessary factor for regeneration of aromatic rice cv. KDML105.

Nanoparticles are well known that materials not only play physico-chemical properties for potential electronics but also apply a role in the organism metabolism (Su *et al.,* 2007; Viana *et al.,* 2010). This is the first study which reported and investigated the effect of nanoparticles on the plant regeneration frequency in aromatic rice cultivar. Interestingly, applications of ZnO nanoparticles showed the low efficiency of regeneration frequency whereas supplementation of TiO_2 nanoparticles in regeneration medium showed the high efficiency of regeneration frequency than the control without nanoparticles. The results in this study also indicated that supplementing ZnO nanoparticles in the regeneration medium did not improve the regeneration frequency and had a toxic role in plant cell while TiO_2 nanoparticles enhanced the regeneration efficiency. The high regeneration frequency was obtained in regeneration medium supplemented with 25 mg L^{-1} TiO_2 nanoparticles. Zinc is a trace element for plant metabolism but the high dose of zinc is phytotoxic in various plant species (El-Ghamery *et al.,* 2003; Paschke *et al.,* 2006). The evident toxicities of ZnO nanoparticles agree with studies reported in various plant species (Lin and Xing, 2007; López-Moreno *et al.,* 2010). The different dose of ZnO nanoparticles might induced reactive oxygen species accumulation that caused cell damage, phytotoxic, genotoxic and gene expression alteration but the effect of toxic level depend on the differentiation of plant species (Singh *et al.,* 2009; Nair *et al.,* 2010; Soenen *et al.,* 2011). While, the previous research presented TiO_2 nanoparticles activated photosynthetic complexes Rubisco carboxylase activity and nitrogen metabolism in plant cell at a proper concentration which may induce cell differentiation and cell growth (Yang *et al.,* 2006; Zheng *et al.,* 2008). Moreover, TiO_2 nanoparticles assist the water absorption in

Table 4: The effect of ZnO nanoparticles on the percentages of green spots, regeneration frequency and the ratio of the number of seedlings to the number of regenerated calli from the calli of aromatic rice cv. KDML105 cultured on the regeneration stage

ZnO nanoparticles (mg L^{-1})	% Green spots	% Regeneration frequency	The ratio of seedlings/regenerated calli
0	94.54 ±1.25 a	55.46 ±1.25 a	2.54 ±0.04 a
5	88.52 ±1.70 c	48.27 ±1.67 d	2.55 ±0.02 a
25	92.35 ±2.23 b	51.73 ±1.67 b	2.57 ±0.07 a
50	91.23 ±4.06 b	50.62 ±1.07 c	2.55 ±0.02 a

Mean values were taken from average of three replication (n=3). Mean values ±SD followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Duncan's New Multiple Range Test (DMRT)

Table 5: The effect of TiO_2 nanoparticles on the percentages of green spots, regeneration frequency and the ratio of the number of seedlings to the number of regenerated calli from the calli of aromatic rice cv. KDML105 cultured on the regeneration stage

TiO_2 nanoparticles (mg L^{-1})	% Green spots	% Regeneration frequency	The ratio of seedlings/regenerated calli
0	94.54 ±1.25 a	55.46 ±1.25 b	2.54 ±0.04 b
5	94.80 ±1.31 a	55.99 ±1.60 b	2.54 ±0.04 b
25	94.96 ±1.50 a	56.46 ±0.82 a	2.80 ±0.03 a
50	93.41 ±1.78 b	54.88 ±2.00 c	2.53 ±0.03 b

Mean values were taken from average of three replication (n=3). Mean values ±SD followed by the same letters in each column are not significantly different at $P \leq 0.05$ according to Duncan's New Multiple Range Test (DMRT)

plant cell and induce cellular metabolism which could promote the plant growth and differentiation (Zheng *et al.,* 2005). The low and/or appropriate dose of TiO_2 nanoparticles had positive response on plant metabolism (Song *et al.,* 2013). The species, ages and concentration-dependent plants are influenced from the toxic exposition from plant cell (Ma *et al.,* 2010). Moreover, the previous researches reported that Ti have the positive and no toxic effects on plant physiology and morphology (Ghosh *et al.,* 2010; Tlustoš *et al.,* 2005).

Conclusion

The present research concluded that the culture condition and nanoparticles; especially TiO_2 usefully improved the callus induction and plant regeneration of aromatic rice cv. KDML105.

Acknowledgements

The author is grateful to Pathumthani Rice Research Center and Seed Center, Roiet, Office of grain Rice Department, Ministry of Agriculture and Cooperatives for providing of rice seeds. This research was kindly supported by Office of the National Research Council of Thailand (NRCT).

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(Received 11 November 2014; Accepted 13 April 2015)