Application of activated charcoal and nanocarbon to callus induction and plant regeneration in aromatic rice (*Oryza sativa* L.)

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Abstract

The investigations of nanotechnology with the application on agricultural products also have been few reported, especially the plant regeneration. The effects of activated charcoal and nanocarbon on the callus induction and plant regeneration of aromatic rice were studied. Activated charcoal was added into the callus induction and regeneration medium. The presence of activated charcoal in the callus induction medium (100–500 mg L\(^{-1}\)), activated charcoal significantly reduced the percentage of the callus induction and biomass accumulation (fresh weight, dry weight and size). Whereas, the regeneration medium supplemented with 100 mg L\(^{-1}\) of activated charcoal showed the highest percentage of plant regeneration (61.90%) and the ratio of the number of seedlings to the number of regenerated calli (RSR; 3.06) that derived from the callus induction medium (without activated charcoal). Moreover, the induced calli derived from the callus induction medium supplemented with nanocarbon at 5 mg L\(^{-1}\) showed the highest percentage of callus induction (94.70%), the percentage of green spots (95.83%), the percentage of plant regeneration (60.42%) and the RSR (3.12) when transferred the calli into the regeneration medium (without nanocarbon). After that, nanocarbon was also added into the regeneration medium. The percentage of green spots (96.08%), the percentage of plant regeneration (62.75%) and the RSR (3.16) obtained from the regeneration medium supplemented with 20 mg L\(^{-1}\) of nanocarbon showed the highest values. This experiment showed that the optimum concentration of activated charcoal and nanocarbon had potential to enhance the callus induction and plant regeneration frequencies in tissue culture medium of aromatic rice.

1. Introduction

Recently, the researches in nanoscience and nanotechnology have been applied to a variety of scientific areas, ranging from electronics, energy, medicine and agriculture [1-4]. Unfortunately, there are a few studies that apply the nanomaterials in agricultural feature. Due to the unique properties of nanomaterials, many of scientists use nanomaterials to improve the agricultural techniques [5,6]. Mostly, researches and reliable information study the effect of nanomaterials on plant physiology and plant morphology at the seedling stage [7,8]. A nanomaterial, especially nanocarbon is used in material science, electronic and physical researches. Moreover, nanocarbon also applies in biological researches [9,10]. Comparing to the researches of nanomaterials and nanocarbon in plant science, the application of nanomaterials used in plant cell or tissue culture for plant regeneration and differentiation has some limitation [11,12].

Rice is known as one of the staple food in the world and the rice consumers are increased every year. In contrast, the rice productions are decreased due to biotic and abiotic stresses problems during rice cultivation [13,14]. The development of *in vitro* culture or tissue culture technique that collaborated with genetic transformation and/or micropropagation is a key factor to solve the problems of rice production. In addition, an efficient regeneration frequency is a first step that leads to a successful application of gene technology [15,16]. Success of plant tissue culture is depending on the interaction between plant variety, explants used and culture medium [17,18]. Culture medium requires organic supplements that added in a growth medium such as mineral ions, carbon source and vitamins [19,20]. The previous researches have been published and shown that the exogenous application of amino acid, antioxidant substances, plant growth regulators, and desiccation treatment could improve regeneration frequency in rice tissue culture [21-25]. However, few researches have been examined the effect of nanomaterials on plant regeneration frequency, especially *indic*a rice and aromatic rice varieties. Therefore, the knowledge on the effect of various supplements on regeneration medium.
is very significance [26,27]. It is necessary to improve specific regeneration medium for each plant species and variety.

Due to activated charcoal has a unique property, it is able to reduce the oxidation of phenolic compounds. It is used in tissue culture media due to it can promote and inhibit plant growth in in vitro experiment [28–30]. As we know that the phenolic compounds can cause the cell death and inhibit the regeneration [30]. Activated charcoal serves as phenolic compounds adsorption exudates in plant cell culture so it is often used to improve cell growth and development in plant cell differentiation [31,32]. On the contrary, disadvantage of activated charcoal was also reported. Activated charcoal can absorb exogenous hormone and organic chemicals from the culture medium [33,34]. Therefore, the adequate level of exogenous hormone and organic chemicals including activated charcoal in the culture medium is necessary for plant tissue culture. According to the advantage of activated charcoal and the application of nanocarbon, this experiment was studied the effect of activated charcoal and nanocarbon that optimization of the appropriate level of them on the callus induction and plant regeneration frequencies of aromatic rice variety Khao Dawk Mali 105 (KDML105).

2. Materials and methods

2.1. Plant samples

Aromatic rice (Oryza sativa L. cv. Khao Dawk Mali 105) seeds were dehusked and sterilized by 70% ethanol for 2–3 min, followed by 5% (v/v) commercial bleach (5.25% sodium hypochlorite) for 40 min, and 30% (v/v) commercial bleach for 3–2 min, followed by 5% (v/v) commercial bleach (5.25% sodium hypochlorite) for 40 min, and 30% (v/v) commercial bleach for 30 min. Then the seeds were thoroughly rinsed with sterile-distilled water 5–6 times [12,35].

2.2. The effect of activated charcoal on the callus induction and plant regeneration frequencies

The sterilized seeds were transferred to the basal NB medium [36] that contained 2 mg L⁻¹ of 2,4-D, 500 mg L⁻¹ of glutamine, 500 mg L⁻¹ of proline, 8 g L⁻¹ of agar and 30 g L⁻¹ of maltose (NMI medium). Supplement of different amount of activated charcoal (average size 10.85 ± 5.40 μm; Sigma-Aldrich, USA) at 100, 200, 300, 400 and 500 mg L⁻¹ were varied into NMI medium. The pH of the medium was adjusted to 5.6–5.8. Then the seeds were cultured under 25 ± 2 °C and 1000 lux light intensity condition for 4 weeks. The percentage of green spots, plant regeneration and the ratio of the number of seedlings to the number of regenerated calli (RSR) were collected for evaluation. The percentage of the callus induction, green spots, plant regeneration and the ratio of the number of seedlings to the RSR were calculated as follow:

\[ \text{No. of seeds induced calli} \]
\[ \text{No. of seeds cultured} \] × 100

\[ \text{No. of callus induced green spots} \]
\[ \text{No. of seeds cultured} \] × 100

\[ \text{No. of callus induced shoot buds (plants)} \]
\[ \text{No. of seeds cultured} \] × 100

\[ \text{No. of callus induced shoot buds (plants)} \]
\[ \text{No. of callus induced shoot buds (plants)} \]

2.3. The effect of nanocarbon on the callus induction and plant regeneration frequencies

The sterilized seeds were transferred to NMI medium contained various amount of nanocarbon (carbon black; average size 52.50 ± 8.90 nm; Global Chemical Co., Ltd., Thailand) at 5, 25, 50 and 100 mg L⁻¹, respectively. The morphology and size of nanocarbon were characterized by transmission electron microscope (TEM, JEM-2010) and particle analyzer (Delsa™Nano C). The pH of the medium was adjusted to 5.6–5.8. Then the seeds were cultured under 25 ± 2 °C in dark condition for 3 weeks. The percentage of the callus induction, fresh weight, dry weight and size were evaluated after 3 weeks of incubation.

To study the effect of nanocarbon on the plant regeneration frequency, the appropriate concentration of nanocarbon in NMI medium from previous section were used. The induced calli were desiccated on sterilized filter (Whatman™ No.1) paper in dark condition for 1 week before transferred to NMR supplemented with 10, 20, 30, 40 and 50 mg L⁻¹ of nanocarbon. The pH of the medium was adjusted to 5.6–5.8. The calli were cultured under 25 ± 2 °C and 1000 lux light intensity condition for 4 weeks. The percentage of green spots, plant regeneration and the RSR were collected for evaluation.

2.4. Data and statistical analysis

All experiments in this study were designed in Completely Randomized Design (CRD) with five replications in each
induced in the callus induction medium without activated charcoal). The results showed that the percentage of plant regeneration belonged to NMR medium supplemented with activated charcoal at 0, 100, 200, 300, 400 and 500 mg L$^{-1}$, respectively (Table 2 and Figure 2).

From the results showed that NMR medium supplemented with 100 mg L$^{-1}$ of activated charcoal enhance the percentage of plant regeneration. Activated charcoal acts as the phenolic compounds absorption in tissue culture medium. Due to the phenolic compounds are able to destroy the cell wall and reduce the plant regeneration frequency. Moreover, activated charcoal is potential absorption various useful substances such as mineral nutrients, growth hormones and plant growth regulators in plant tissue culture medium [37]. The pH of tissue culture medium has been increased when activated charcoal is present. Drifting pH of tissue culture medium is improper condition for plant growth and differentiation [38]. Therefore, the optimum concentration of activated charcoal could be reduce the oxidation of phenolic compounds in plant regeneration media and enhance the plant regeneration frequency [39,40].

Moreover, nanocarbon was added to the callus induction medium to enhance the callus induction frequency.

### Table 1. The percentage of the callus induction, fresh weight, dry weight and callus size of 3 weeks calli derived from NMI medium supplemented with activated charcoal at 0, 100, 200, 300, 400 and 500 mg L$^{-1}$.

| Activated charcoal concentrations (mg L$^{-1}$) | Percentage of Callus induction (%) | Fresh weight (mg) | Dry weight (mg) | Callus size (cm) |
|-----------------------------------------------|-----------------------------------|------------------|----------------|------------------|
| 0                                            | 93.56 ± 0.62 a                    | 92.41 ± 6.23 a   | 10.84 ± 1.52 a | 0.93 ± 0.08 a    |
| 100                                          | 92.03 ± 0.60 b                    | 76.17 ± 4.00 b   | 9.16 ± 0.75 b  | 0.76 ± 0.05 b    |
| 200                                          | 89.39 ± 0.98 c                    | 67.88 ± 2.52 c   | 7.07 ± 0.69 c  | 0.60 ± 0.08 c    |
| 300                                          | 87.32 ± 1.43 d                    | 57.08 ± 1.81 d   | 4.87 ± 0.51 d  | 0.57 ± 0.05 c    |
| 400                                          | 82.95 ± 0.60 e                    | 50.95 ± 4.43 e   | 3.60 ± 0.47 e  | 0.51 ± 0.07 d    |
| 500                                          | 80.68 ± 0.43 f                    | 40.28 ± 1.87 f   | 2.43 ± 0.35 f  | 0.44 ± 0.05 e    |

Notes: Mean values were taken from average of five replication ($n = 5$). Mean values ±SD followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.

### Table 2. The percentage of green spots, the percentage of plant regeneration and the ratio of the number of seedlings to the number of regenerated calli of 3-week-old calli derived from NMI medium (without activated charcoal) were transferred to the regeneration medium that supplemented with various amount of activated charcoal; without activated charcoal, 100, 200, 300, 400 and 500 mg L$^{-1}$ for 4 weeks.

| Activated charcoal concentrations (mg L$^{-1}$) | Percentage of green spots (%) | Percentage of plant regeneration (%) | Ratio of no. of seedlings to no. of regenerated calli |
|-----------------------------------------------|-------------------------------|--------------------------------------|------------------------------------------------------|
| 0                                            | 96.53 ± 0.82 a                | 59.77 ± 0.90 b                       | 3.02 ± 0.03 a                                       |
| 100                                          | 96.43 ± 0.80 a                | 61.90 ± 0.84 a                       | 3.06 ± 0.03 a                                       |
| 200                                          | 95.40 ± 0.75 b                | 56.32 ± 0.88 c                       | 2.84 ± 0.03 b                                       |
| 300                                          | 94.25 ± 0.65 b                | 55.17 ± 0.77 c                       | 2.73 ± 0.03 b                                       |
| 400                                          | 93.10 ± 0.78 c                | 51.72 ± 0.79 d                       | 2.60 ± 0.04 c                                       |
| 500                                          | 93.10 ± 0.82 c                | 51.72 ± 0.82 d                       | 2.53 ± 0.04 c                                       |

Notes: Mean values were taken from average of five replication ($n = 5$). Mean values ±SD followed by the same letters in each column are not significantly different at $p \leq 0.05$ according to Duncan's multiple rang test.
Nanocarbon was spherical shape and the average size was approximately 40–60 nm (Figure 1(A)). The induced calli derived from NMI medium supplemented with nanocarbon at 5 mg L⁻¹ showed higher the percentage of callus induction (94.70%) than calli derived from NMI medium supplemented with other amount of nanocarbon concentration (0, 25, 50 and 100 mg L⁻¹ nanocarbon). The result of the fresh weight, dry weight and callus size of NMI medium supplemented with nanocarbon at 5 mg L⁻¹ were 116.44 mg, 16.26 mg and 1.10 cm, respectively. The percentage of callus induction was 93.75, 90.91, 88.19 and 86.51% in calli derived from NMI medium supplemented with nanocarbon at 0, 25, 50 and 100 mg L⁻¹, respectively. The fresh weight of calli were 92.51, 135.86, 165.73 and 198.59 mg, the dry weight of calli were 10.89, 16.86, 20.23 and 22.09 mg and callus size were 0.93, 1.17, 1.21 and 1.34 cm from the calli derived from NMI medium supplemented with nanocarbon at 0, 25, 50 and 100 mg L⁻¹, respectively (Table 3 and Figure 1(B)).

The induced calli derived from NMI medium supplemented with 0, 5, 25, 50 and 100 mg L⁻¹ of nanocarbon were transferred to the regeneration medium (NMR medium). The calli derived from NMI medium supplemented with 5 mg L⁻¹ of nanocarbon showed the highest percentage of green spots (95.83%), the percentage of plant regeneration (60.42%) and the RSR (3.12) than calli derived from NMI medium supplemented with other amount of nanocarbon concentration (0, 25, 50 and 100 mg L⁻¹). The percentage of green spots were 95.24, 93.55, 91.67 and 91.67%, the percentage of plant regeneration were 59.52, 55.91, 50.00 and 39.58% and the RSR were 3.10, 2.88, 2.54 and 2.34 from the calli derived from NMI medium supplemented with nanocarbon at 0, 25, 50 and 100 mg L⁻¹, respectively (Table 4).

The percentage of plant regeneration increased upon exposure to low concentration of nanocarbon in the callus induction medium. From this experiment result, the calli derived from NMI medium supplemented with 5 mg L⁻¹ of nanocarbon can induce more embryogenic callus than other medium as it gave the highest percentage of callus induction.

![Image](A) Transmission electron microscope (TEM) image of nanocarbon and (B) appearance of calli derived from NMI medium supplemented with nanocarbon at 0, 5, 25, 50 and 100 mg L⁻¹.

### Table 3. The percentage of the callus induction, fresh weight, dry weight and callus size of 3 weeks calli derived from NMI medium supplemented with nanocarbon at 0, 5, 25, 50 and 100 mg L⁻¹.

| Nanocarbon concentration (mg L⁻¹) | Percentage of Callus induction (%) | Fresh weight (mg) | Dry weight (mg) | Callus size (cm) |
|----------------------------------|-----------------------------------|-------------------|----------------|-----------------|
| 0                                | 93.75 ± 0.57 b                    | 92.51 ± 7.10 e    | 10.89 ± 1.89 d | 0.93 ± 0.07 d   |
| 5                                | 94.70 ± 0.86 a                    | 116.44 ± 9.51 d   | 16.26 ± 2.59 c | 1.10 ± 0.10 c   |
| 25                               | 90.91 ± 0.69 c                    | 135.86 ± 10.48 c  | 16.86 ± 4.31 c | 1.17 ± 0.08 c   |
| 50                               | 88.19 ± 0.60 d                    | 165.73 ± 10.66 b  | 20.23 ± 2.10 b | 1.21 ± 0.07 b   |
| 100                              | 86.51 ± 0.69 e                    | 198.59 ± 10.32 a  | 22.09 ± 4.30 a | 1.34 ± 0.13 a   |

Notes: Mean values were taken from average of five replication (n = 5). Mean values ±SD followed by the same letters in each column are not significantly different at p ≤ 0.05 according to Duncan’s multiple range test.

### Table 4. The percentage of green spots, the percentage of plant regeneration and the ratio of the number of seedlings to the number of regenerated calli of 3-week-old calli derived from NMI medium that supplemented with various amount of nanocarbon; without nanocarbon, 5, 25, 50 and 100 mg L⁻¹ and transferred to the regeneration medium (without nanocarbon) for 4 weeks.

| Nanocarbon concentration (mg L⁻¹) | Percentage of green spots (%) | Percentage of plant regeneration (%) | Ratio of no. of seedlings to no. of regenerated calli |
|-----------------------------------|------------------------------|--------------------------------------|------------------------------------------------------|
| 0                                 | 95.24 ± 0.92 a               | 59.52 ± 0.86 b                       | 3.10 ± 0.05 a                                        |
| 5                                 | 95.83 ± 0.90 a               | 60.42 ± 0.94 a                       | 3.12 ± 0.05 a                                        |
| 25                                | 93.55 ± 0.84 b               | 55.91 ± 0.93 c                       | 2.88 ± 0.03 b                                        |
| 50                                | 91.67 ± 0.89 c               | 50.00 ± 0.95 d                       | 2.54 ± 0.04 c                                        |
| 100                               | 91.67 ± 0.84 c               | 39.58 ± 0.96 e                       | 2.34 ± 0.03 c                                        |

Notes: Mean values were taken from average of five replication (n = 5). Mean values ±SD followed by the same letters in each column are not significantly different at p ≤ 0.05 according to Duncan’s multiple range test.
of plant regeneration. Even though, high concentration of nanocarbon enhanced high callus size and biomass [41]. The previous researches reported that nanocarbon (carbon nanotube) was able to increase the growth rate of tobacco cell culture [11] and mustard plant [42].

This is the first report that used nanocarbon as a supplement in the plant regeneration medium for investigation of cell differentiation in aromatic rice. Nanocarbon at 0, 10, 20, 30, 40 and 50 mg L^-1 were added into the plant regeneration medium to enhance the plant regeneration frequency. The enhanced level of the plant regeneration frequency was observed in treatment received 20 mg L^-1 nanocarbon when compared to other treatments (0, 10, 30, 40 and 50 mg L^-1 nanocarbon). From this experiment result showed that the highest values of the percentage of green spots (96.08%), the percentage of plant regeneration (62.75%) and the RSR (3.16) were obtained from NMR medium supplemented with 20 mg L^-1 of nanocarbon, followed by NMR medium supplement with 0, 10, 30, 40 and 50 mg L^-1 of nanocarbon, respectively. The percentage of green spots were 95.24, 95.10, 95.83, 94.79 and 93.33%, the percentage of plant regeneration were 60.00, 61.76, 59.38, 58.33 and 52.22% and the RSR were 3.10, 3.11, 3.11, 3.07 and 3.02 from the calli derived from NMR medium supplemented with nanocarbon at 0, 10, 30, 40 and 50 mg L^-1, respectively (Table 5 and Figure 3).

The previous research reported that the nanocarbon materials have deleterious effect to plant cell at high concentration of nanomaterials. High doses of nanocarbon materials has been reported to be negatively impacted by the production and accumulation of reactive oxygen species (ROS) and necrotic symptoms in plant cells [43,44]. The result revealed that the appropriated concentration of nanomaterials applied can effect to the growth rate of plant cell [41,45]. The optimum

![Figure 2](image1.png)

**Figure 2.** The appearance of plant regeneration derived from NMI medium (without activated charcoal) were transferred to the regeneration medium that supplemented with various amount of activated charcoal; without activated charcoal (A), 100 mg L^-1 (B), 200 mg L^-1 (C), 300 mg L^-1 (D), 400 mg L^-1 (E) and 500 mg L^-1 (F) for 4 weeks.

![Table 5](image2.png)

**Table 5.** The percentage of green spots, the percentage of plant regeneration and the ratio of the number of seedlings to the number of regenerated calli of 3-week-old calli derived from NMI medium that supplemented with 5 mg L^-1 nanocarbon and transferred to the regeneration medium that supplemented with various amount of nanocarbon; without nanocarbon, 10, 20, 30, 40 and 50 mg L^-1 for 4 weeks.

| Nanocarbon concentration (mg L^-1) | Percentage of green spots (%) | Percentage of plant regeneration (%) | Ratio of no. of seedlings to no. of regenerated calli |
|-----------------------------------|------------------------------|-------------------------------------|-----------------------------------------------------|
| 0                                 | 95.24 ± 0.95 b               | 60.00 ± 0.89 c                      | 3.10 ± 0.04 b                                       |
| 10                                | 95.10 ± 0.94 b               | 61.76 ± 0.95 b                      | 3.11 ± 0.03 b                                       |
| 20                                | 96.08 ± 0.89 a               | 62.75 ± 0.89 a                      | 3.16 ± 0.04 a                                       |
| 30                                | 95.83 ± 0.97 a               | 59.83 ± 0.95 c                      | 3.11 ± 0.03 b                                       |
| 40                                | 94.79 ± 0.95 c               | 58.33 ± 0.96 d                      | 3.07 ± 0.03 b                                       |
| 50                                | 93.33 ± 0.99 c               | 52.22 ± 0.96 e                      | 3.02 ± 0.03 c                                       |

Notes: Mean values were taken from average of five replication (n = 5). Mean values ±SD followed by the same letters in each column are not significantly different at p ≤ 0.05 according to Duncan’s multiple rang test.
concentration of nanocarbon might be enhancing the water and/or nutrient transport and the gene regulations involved in cell division, cell wall extension and water transport from environment to plant cell \([11,46–48]\). Aquaporins (water channel) protein level was a key role in plant-water relation and cell development. It was significantly high level in plant cell that treated with carbon nanotube \([11,48,49]\). Moreover the high concentration of nanocarbon is also known to have cytotoxicity to plant cell due to excessive generation of ROS in plant cells. The optimum concentration of nanocarbon was also found to have the positive effect of reducing the accumulation of ROS by stimulation of antioxidant enzyme activities \([50–54]\).

4. Conclusions

This experiment confirmed that the use of activated charcoal and nanocarbon in low concentrations \((100\text{ and }20\text{ mg L}^{-1})\), respectively on the plant regeneration medium could enhance the plant regeneration frequency of aromatic rice. Nanocarbon has more potential than activated charcoal in the callus induction and plant regeneration frequencies. From the result, 5 and 20 mg L\(^{-1}\) of nanocarbon were the optimum concentration for the callus induction and plant regeneration stage for aromatic rice \((\text{Oryza sativa L. cv. Khao Dawk Mali 105})\), respectively. The optimum concentration of nanocarbon supplementation can be used to enhance the potential of the callus induction and plant regeneration frequencies for further plant breeding and/or gene transformation step in the future.

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Disclosure statement

The authors declare that they have no conflict of interest.

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\text{Figure 3. The appearance of plant regeneration derived from NMI medium that supplemented with 5 mg L}^{-1}\text{ nanocarbon and transferred to the regeneration medium that supplemented with various amount of nanocarbon; without nanocarbon (A), 10 mg L}^{-1}\text{ (B), 20 mg L}^{-1}\text{ (C), 30 mg L}^{-1}\text{ (D), 40 mg L}^{-1}\text{ (E) and 50 mg L}^{-1}\text{ (F) for 4 weeks.}
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