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Mustard and Chinese Cabbage Plant Growth Promotion by Optimal-Medium-Cultured *Acinetobacter calcoaceticus* SE370

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This study was aimed to optimize the culture conditions for the growth of *Acinetobacter calcoaceticus* SE370 to produce bioactive substances for plant growth promotion. A basal medium containing 0.3% yeast extract, 0.3% Na_2HPO_4, 0.05% $MgCl_2$, and pH of 7.0 was used as the control. Sources of carbon (glucose, fructose, sucrose, and mannitol), and nitrogen (peptone, tryptone, NH_4Cl, KNO_3, and yeast extract) and different culture temperatures (25 °C, 30 °C, 35 °C and 38 °C) were used for the control medium for *A. calcoaceticus* SE370 culture optimization. The rate of cell growth was higher for the optimal medium containing 1.5% glucose and 1% tryptone in a basal medium at 35 °C when compared to that for the control. The production of malic, citric, succinic, and gibberellic acids was high by *A. calcoaceticus* SE370 strain cultured in the optimal medium. Further, an enhanced level of phosphate solubilization was noted in *A. calcoaceticus* SE370 cultured in the optimal medium. The growth of mustard and Chinese cabbage seedlings significantly increased by using *A. calcoaceticus* SE370 cultured in optimal medium, resulting in increased shoot and root lengths, fresh weight, and chlorophyll content. Thus, an optimal medium composition for *A. calcoaceticus* SE370 culture enhanced the production of plant growth promoting substances, subsequently accentuating plant growth.

Keywords: Bacteria, medium components, organic acid, phosphate solubilization, seedlings.

The use of plant growth promoting microorganisms to improve crop productivity is a major issue in agricultural research. Microorganisms have the ability to produce phytohormones, solubilize insoluble phosphate, and convert complex organic substances into simple forms[1]. Several reports suggest that the application of plant growth promoting bacteria, *Bacillus, Enterobacter, Burkholderia, Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Beijerinckia, Erwinia, Flavobacterium, Rhizobium,* and *Serratia* in soil, can be useful to increase plant growth[2, 3, 4].

Acinetobacter calcoaceticus is present in the skin and throat of a human body as normal flora[5], and is also found in soil as a typical bacterium[6]. In our previous study, *Acinetobacter calcoaceticus* SE370, a bacterial isolate was

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identified as a plant growth-promoting rhizobacterium, which secreted gibberellin (GA), organic acid, and solubilize the phosphate in a culture medium[7, 8]. GA is a plant hormone responsible for seed germination, dormancy, stem elongation, flowering, and senescence. GA producing fungi and bacteria induced plant growth enhancement under unfavorable environment and stressful conditions in many crop plants[1, 8, 9]. It also accentuated phosphate solubilization, by converting insoluble phosphate into soluble phosphate[10]. Phosphorus is essential to protein synthesis, cell division, and development of plants. Inoculation of phosphate solubilizing rhizobacteria in soil synthesizes organic acids to increase the solubilization of phosphate[11]. The low molecular weight organic acids produced by microorganisms and plant root exudates perform many functions such as root nutrient acquisition, mineral weathering, microbial chemotaxis, and detoxification[12]. Moreover, the exogenous application of *A. calcoaceticus* SE370 is considered an eco-friendly plant growth-promoting rhizobacteria (PGPR) for cucumber plant growth[8].

Previous studies have shown that phosphate solubilization and production of organic acid and gibberellin in *A. calcoaceticus* SE370 culture could improve growth of crop plants[7, 8]. From this hypothesis, we aimed to develop the optimal medium for *A. calcoaceticus* SE370 culture to enhance the plant growth promoting metabolites in a culture medium with different sources and concentration of carbon, nitrogen, and temperature.

MATERIALS AND METHODS

Optimization of medium components for *A. calcoaceticus* SE370 culture

A. calcoaceticus SE370 was isolated from the soil samples collected from Geongbuk province, Korea, and identified using 16S rDNA sequence and phylogenetic analysis[7]. The basal medium containing yeast extract of 0.3% (nitrogen source), glucose (1.0%) (carbon source) Na_2HPO_4 (0.3%), $MgCl_2$ (0.05%), and pH of 7.0, with an incubation temperature of 30 °C was considered as the control medium for the experiment. For optimization of the medium components, the various sources of carbon (1%) and nitrogen (0.3%), such as glucose, fructose, sucrose, mannitol, peptone, tryptone, NH_4Cl, KNO_3, and yeast extract were added separately to the basal medium, except for 1% glucose and 0.3% yeast extract. *A. calcoaceticus* SE370 culture (10^8 cfu/ml) was inoculated in the medium, and cell growth was measured at 600 nm by using a T60 UV/VIS spectrophotometer (Oasis scientific. Inc., USA), between 3 and 48 h. In the preliminary study, *A. calcoaceticus* SE370 cell growth was higher in medium containing 1% sucrose and 0.3% tryptone. Different concentrations of sucrose and tryptone in the range of 0.5–2.0% and 0.3–1.0%, respectively, were tested and the cell growth was measured.

For determination of the optimal temperature, *A. calcoaceticus SE370* was inoculated in a medium containing Na_2HPO_4 0.3%, $MgCl_2$ 0.05%, sucrose 1.5%, and tryptone 1.0%, with a pH of 7.0. The culture was incubated at 25 °C, 30 °C, 35 °C, and 38 °C, and cell growth was measured between 3 and 48 h at 600 nm.

Organic acid, free phosphate and gibberellin content in culture medium

A. calcoaceticus SE370 culture filtrate was separated by 0.22- µm Millipore filter, and 10 µl of filtrate was injected into HPLC (Model: Waters 600E) equipped with a Refractive Index Detector (Model: Waters 410; Column: RSpak KC-811 [8.0 × 300 mm]; Eluent: 0.1% H_3PO_4 in H_2O; Flow rate: 1.0 $ml \cdot min^{-1}$; Temperature: 40 °C). The malic, citric, and succinic acid levels were determined and calculated based on the peak values of authentic standards of malic, citric, and succinic acid purchased from Sigma Aldrich, USA[8].

Phosphate solubilizing ability of *A. calcoaceticus* SE370 was tested by the inoculation of bacteria into 100 ml of optimum medium containing tricalcium phosphate at 35 °C, and soluble phosphate and pH was measured after 24 and 48 h; pH of the medium was recorded with a pH meter equipped with glass electrode. Quantitative spectrophotometric analysis of the soluble phosphate was performed according to the method described by King[13].

Gibberellic acid was extracted from *A. calcoaceticus* SE370 cultured in optimal and basal media according to the method of Lee et al.[14]. The extracted GA was injected to GC (Hewlett-Packard 6890, 5973N Mass Selective Detector) with HA-1 capillary column (30 m × 0.25 mm i.d. 0.25-µm film thickness) at an oven temperature of 60 °C for 1

min, after which the temperature was increased at 15 °C·min^{-1} to 200 °C, followed by 5 °C·min^{-1} to 285 °C. Helium carrier gas was used and maintained at a head pressure of 30 kPa. The GC was contacted to a Mass Selective Detector with an interface and source temperature of 280 °C, an ionizing voltage of 70 eV and a dwell time of 100 ms. The endogenous GA_4 content was calculated from peak area ratios of 284/286.

Plant growth promotion in mustard and Chinese cabbage

Mustard and Chinese cabbage seeds were sown in autoclaved plastic pots under controlled greenhouse conditions at 30 ± 2 ºC. The seedlings were treated with 5 ml of bacterial suspension after 2 weeks of sowing, and growth attributes such as shoot and root lengths, fresh weight, and chlorophyll contents were recorded at 21 days of culture. The chlorophyll content of fully expanded leaves was analyzed using a chlorophyll meter (Minolta Co., Ltd, Japan).

Statistical analysis

All statistical analyses were performed using sigma plot software and the mean values were calculated using Duncan's multiple range test at $P < 0.05$ (ANOVA SAS release 9.1; SAS Cary, NC, USA).

RESULTS AND DISCUSSION

The culture medium composition and condition influence cell growth and secretion of bioactive metabolites of bacteria. In order to optimize the culture medium components of *A. calcoaceticus* SE370, the effects of various sources of carbon (1%), nitrogen (0.3%), and incubation temperature (25–38 °C) were studied from 3 to 48 h (Fig. 1). Monosaccharides (glucose and fructose), a disaccharide (sucrose), and a sugar alcohol (mannitol) were used as the carbon sources to identify a suitable carbohydrate that would be an ideal source of energy for *A. calcoaceticus*. Sugar alcohol such as mannitol, which is produced from the hydrogenation of fructose, reduced the cell growth of *A. calcoaceticus* SE370 (Fig. 1a). The uptake of fructose by bacteria from the culture medium enters the bacterial metabolism system through glycolysis and stimulates the shutdown metabolism of bacterial cells[15, 16]. The concentration of mannitol controls osmotic potential in the culture medium, and hence, induces osmotic stress in plants[17]. However, the rate of bacterial cell growth was higher in glucose followed by sucrose-containing media. The enzyme sucrase is necessary to breakdown sucrose molecule into glucose and fructose in bacterial cells. Glucose is a quick energy source for all living organisms. In this study, various glucose concentrations (0.5–2.0%) were used to find the optimum concentration for *A. calcoaceticus* SE370 cell growth (Fig. 1b), which was observed at 1.5% glucose concentration. *A. calcoaceticus* SE370 seemed to have utilized glucose more than the other carbon sources.

The bacterial medium supplemented with nitrogen compounds (0.3%), peptone, tryptone, NH_4Cl, KNO_3, and yeast extract were tested to identify the optimum nitrogen source for *A. calcoaceticus* cell growth. The bacterial growth rate was similar in all sources at 3 and 6 h of incubation but varied after 6–48 h of culture (Fig. 1c). The medium containing peptone and NH_4Cl showed lower rate of cell growth when compared to that containing other sources. Nitrogen compounds were added to the medium to synthesize amino acids that form the protein and bases for nucleic acids. Peptone, which is hydrolyzed protein, acted as a nutrient in the culture medium. *A. calcoaceticus* SE370 seemed to absorb

Table 1. Plant growth promoting effect of *Acinetobacter calcoaceticus* SE370 on mustard and Chinese cabbage. Each value is the mean + standard error (SE) of ten replicates per treatment.

Crop	Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Chlorophyll (SPAD)
Mustard	Basal medium	9.8 ± 1.3	12.1 ± 3.4	2.64 ± 1.12	28.5 ± 5.4
	Optimum medium	11.2 ± 1.5	14.3 ± 2.5	3.61 ± 2.42	32.4 ± 4.8
Chinese cabbage	Basal medium	8.7 ± 1.3	12.4 ± 2.1	3.44 ± 1.04	30.1 ± 2.3
	Optimum medium	11.8 ± 1.8	15.3 ± 2.9	4.88 ± 1.12	33.4 ± 3.5

less amount of nitrogen after the breakdown of peptone and the inorganic nitrogen compound, NH_4Cl. Moderate cell growth of *A. calcoaceticus* SE370 was detected in medium inoculated with KNO_3. Tryptone and yeast extract containing culture medium significantly enhanced bacterial cell growth with the fastest cell growth rates observed at 12 and 18 h. Yeast extract containing glutamic acid and tryptone offer a mixture of peptides that are good sources of nitrogen and easily absorbed by bacteria. The use of different concentrations of tryptone revealed that 1%

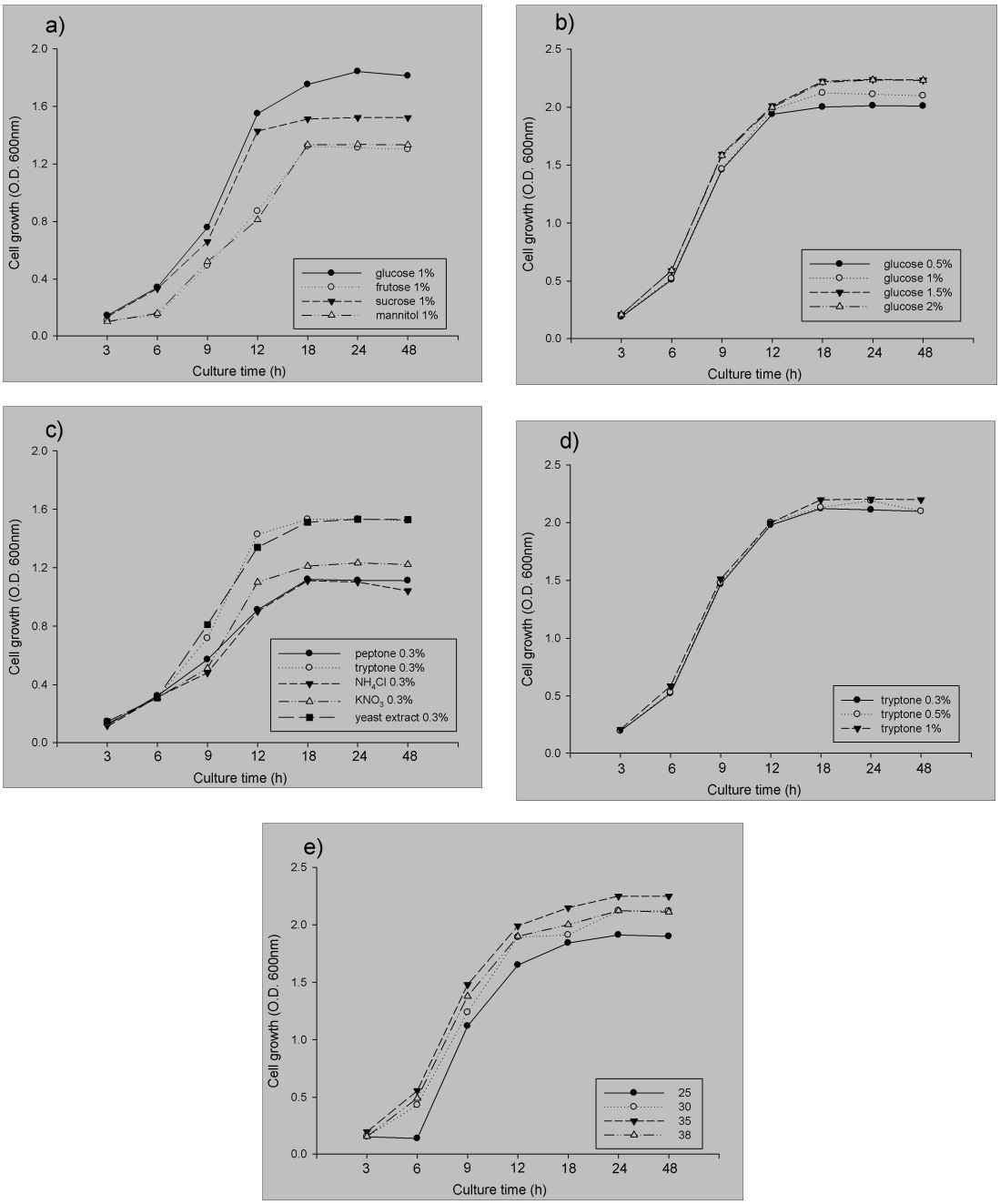

Fig. 1. Effect of various carbon and nitrogen sources, and temperature on *Acinetobacter calcoaceticus* SE370 culture. Each value is the mean + standard error (SE) of three replicates per treatment

tryptone addition to the medium would promote cell growth of *A. calcoaceticus* SE370 (Fig. 1d).

Environmental conditions are also important in determining bacterial cell growth. In this study, a temperature range of 25–38 °C was used to find the optimum temperature for *A. calcoaceticus* cell growth (Fig. 1e). The growth rate of bacterium was the lowest at 25 °C followed by 30 °C and 38 °C, respectively. The maximum cell growth was observed at 35 °C. Temperature affects the structure and function of membrane lipids in organisms[18]. Higher (38 °C) or lower (25 °C and 30 °C) temperatures could degrade the membrane lipids of *A. calcoaceticus* SE370.

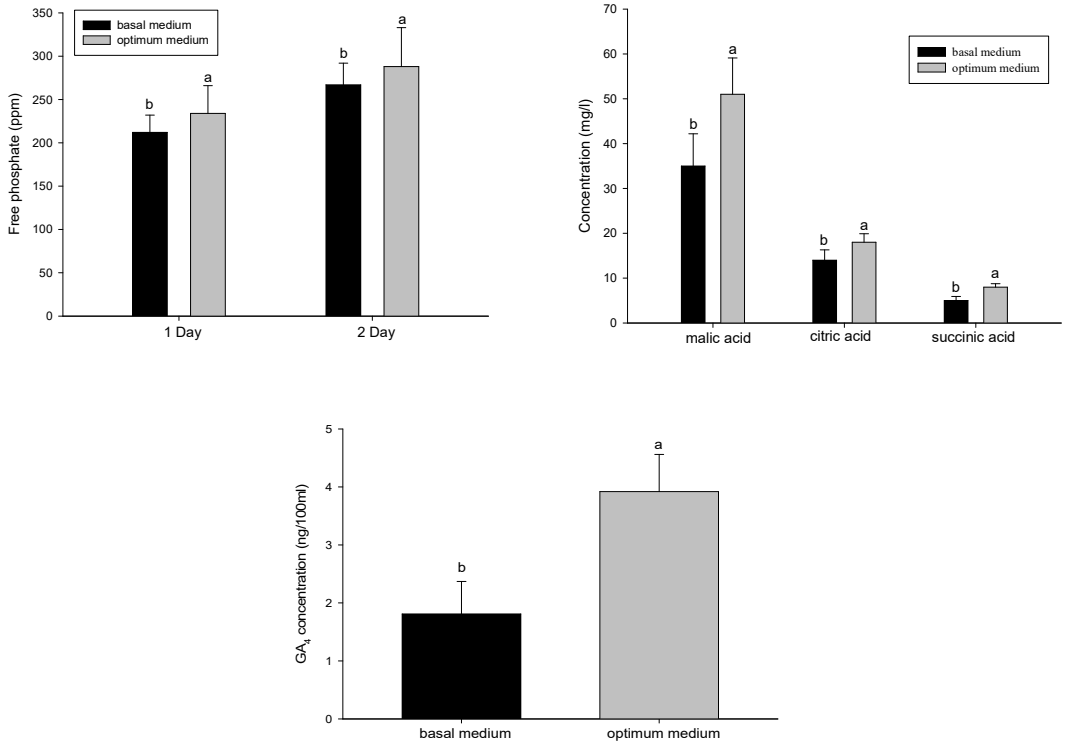

Fig. 2. Phosphate solubilization, production of organic acid and gibberellic acid in *Acinetobacter calcoaceticus* SE370 culture medium. Each value is the mean + standard error (SE) of three replicates per treatment. Mean values followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test

Fig. 3. Effect of *Acinetobacter calcoaceticus* SE370 in culture medium on mustard and Chinese cabbage plants

Phosphate solubilization ability of *A. calcoaceticus* SE370 was accentuated in optimum medium containing glucose (1.5%) and tryptone (1%) as seen from the increased accumulation of free phosphate in the culture medium (Fig. 2a). Gaur[19] and Pallavi and Gupta[20] reported that glucose supplemented in the culture medium was found to be the best carbon source for phosphate solubilization by *Pseudomonas striata* and *Pseudomonas lurida,* respectively. The different nitrogen sources also influenced the phosphate solubilization ability of *Aspergillus awamori* S19[21]. The favorable sources of carbon and nitrogen readily promoted active proliferation of fungi and produced organic acids, which in turn strongly influenced phosphate solubilization[22].

In fungal culture, the production of organic acids such as citric, succinic, and malic acids is associated with inorganic phosphate solubilization[23]. *A. calcoaceticus* SE370 grown in optimal medium showed the production of significant level of malic, citric, and succinic acids (Fig. 2b). Thus, the fungi that produced organic acids may solubilize inorganic phosphate through the release of acidic protons[24].

Plant growth stimulating metabolites secretion from bacteria is well known. In a previous study, it was found that *A. calcoaceticus* SE370 was able to produce gibberellin in nutrient broth medium[7], showing that optimum medium components are necessary for increasing gibberellin production. The levels of GA_4, a bioactive gibberellin, was measured to find out the importance of medium composition for the production of gibberellin, and the result of *A. calcoaceticus* SE370 cultured in an optimal medium showed possible enhancement of GA_4 compared to that cultured in a basal medium (Fig. 2c).

Chinese cabbage is a cool-season crop that has been affected by elevated CO_2 level and temperature[25]. An optimal culture medium promotes plant and bacterial interactions as seen from the results of bacterial treatment on mustard and Chinese cabbage. The shoot and root lengths and fresh weight of mustard and Chinese cabbage were increased by *A. calcoaceticus* SE370 cultured in the optimal medium (Table 1 and Fig. 3), and this growth promotion could be because of increased GA_4 levels in the culture medium. To further validate this hypothesis, the plant growth promoting characters of *A. calcoaceticus* SE370 was confirmed by analyzing the photosynthetic pigment, chlorophyll. The optimum-medium culture of *A. calcoaceticus* SE370 increased the chlorophyll content in mustard and Chinese cabbage plants. Our study results are in accordance with the findings of Dawwam et al.[26], who reported that *Bacillus cereus* and *Achromobacter xylosoxidans* inoculation increased the chlorophyll content in sweet potato.

In conclusion, an optimal medium containing 1.5% glucose, 1% tryptone along with other basal medium salt at 35 °C enhanced the rate of *A. calcoaceticus* SE370 cell growth. The production of malic, citric, succinic, and gibberellic acids was higher in *A. calcoaceticus* SE370 cultured in optimal medium in comparison to that by using the basal medium, which improved the growth of mustard and Chinese cabbage plants. The results of this study suggest that detection of optimal nutritional contents and their concentrations for the culture preparation of a bacterial bio-inoculant, *A. calcoaceticus* SE370, is vital to enhance the production of plant growth-regulating metabolites that promote plant growth.

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