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Effects of Growth Regulators and Organic Nitrogen Sources on the Production of Heavy Chain Immunoglobulin G in Suspension Cultures of Transgenic Tobacco Cells

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Abstract To enhance the production of heavy chain immunoglobulin G (HC IgG) in the suspension cultures of transgenic tobacco cells (*Nicotiana tabacum*), the effects of adding various cytokinins (i.e., growth regulators) and organic nitrogen sources to culture media were investigated. Four different cytokinins including kinetin, isopentenyladenine (IPA), 6-benzylaminepurine (BA), and zeatin were tested with or without dichlorophenoxyacetic acid (2,4-D), which is a typical growth regulator supplemented in the standard Murashige and Skoog (MS) medium. The productivity of intracellular HC IgG was increased by 36 and 42%, compared to the control, especially when IPA (2 mg/l) or BA (0.2 mg/l) was added to the media in the presence of 2,4-D, respectively. In the study of organic nitrogen sources, addition of each casein hydrolysate and tryptone to the culture media at a final concentration of 0.01 and 1 g/l, respectively, increased the productivity of HC IgG as much as 68 and 67%, respectively, in comparison with the control, which was is MS medium without supplementation of any organic nitrogen sources. This study shows that the optimization of media composition could offer significant improvements in the production of foreign proteins in the suspension cultures of transgenic plants.

Key words: *Nicotina tabacum*, transgenic plant, immunoglobulin G, growth regulator, organic nitrogen

In the past decade, significant progress has been made in expressing the genes of various foreign animal proteins in plants [7, 25, 28]. There are many advantages in using plant systems to produce foreign animal proteins, compared to microbial or animal cells [11]: the low production costs, the low risk of contamination, and the correct posttranslational protein modification [3]. Therefore, the strategy of mammalian protein production using transgenic plants, which aims to commercially manufacture antibodies, hormones, and enzymes, is recently receiving much attention [10, 21, 24, 27].

In the growth and development of plants, cytokinins such as kinetin, isopentenyladenine (IPA), 6-benzylaminepurine (BA), and zeatin are known to play important roles [22]. Kinetin was found to effectively stimulate the growth of *Cucumis sativus* [5], and zeatin and IPA also promoted the root growth of *Pinus pinea* [1]. More specifically, it was reported that cytokinins stimulate the production of secondary metabolites in cell cultures of various plant species [2, 18, 19, 20]. Another important role of cytokinins is the stimulatory effect on protein synthesis in many plants [5, 12, 17].

Plant cells are capable of utilizing a wide range of nitrogen sources such as urea, nitrate, glutamine, glutamate, casein hydrolysate, and amino acid mixtures [23, 26]. Generally, protein hydrolysates produced by enzymes are preferable nitrogen sources, because all the amino acids are well preserved in the hydrolysates. For example, casein hydrolysate appears to be an effective nitrogen source in embryo cultures, by supplying amino acids and optimizing the osmolarity in culture media [4, 8, 29]. Therefore, organic nitrogen sources including protein hydrolysates are also considered to be an important factor for the accumulation of foreign proteins in transgenic plant cell cultures.

To enhance the commercial production of foreign proteins by plant cell cultures, which are carried out in large scale, not only the molecular biological methods (e.g., strong promoter) but also the conventional approaches (e.g., media composition and cultivation conditions) are important [24]. However, the effects of medium composition and growth

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regulators on foreign protein production by transgenic plants have not yet been extensively studied. In the present work, the effects of cytokinins and nitrogen sources on the foreign protein production by transgenic *Nicotiana tabacum* were investigated. Heavy chain immunoglobulin G (HC IgG), which is still in high market demand for clinical and research purposes, was chosen as the model foreign protein. To improve the production of HC IgG, four different cytokinins (including kinetin, IPA, BA, and zeatin), and four different organic nitrogen sources (including yeast extract, peptone, casein hydrolysate, and tryptone) were evaluated.

MATERIALS AND METHODS

Transgenic Cell Line

The transgenic tobacco cell line (*N. tabacum*) was genetically modified to express murine HC IgG as described previously [14]. The gene of murine HC IgG in the tobacco cells is specific for *p*-azophenylarsonate [6], and was placed downstream of the CaMV 35S promoter and upstream of the T-DNA transcript 7 gene terminator. The selection of a cell line producing a high level of HC IgG was carried out on the modified Murashige and Skoog (MS) medium (described below) containing cefotaxime and kanamycin.

Stock Suspension Cultures

The transgenic *N. tabacum* cells were cultivated in MS medium [16] supplemented with 30 g of sucrose/l, 0.18 g of KH_2PO_4/l, 1 mg of thiamine HCl/l, and 0.2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D)/l as a growth regulator. The medium pH was adjusted to 5.8 before autoclave at 121°C for 15 min. To prevent contamination during cultivation, cefotaxime and kanamycin were microfiltered (0.2 mm), and added to the medium at concentrations of 100 and 50 mg/l, respectively, before inoculation. The transgenic cells were incubated in 250-ml Erlenmeyer flasks containing 60 ml of the modified MS medium at 28°C with shaking at 120 rpm. To maintain the transgenic cell line, 15 ml of suspension cultures were transferred to fresh media every week.

Measurement of Cell Growth

Two samples of approximately 1.0 ml each were taken daily from each well-agitated suspension culture under aseptic condition. The samples were transferred to preweighed microcentrifuge tubes, and centrifuged at 10,000 ×g for 5 min. The wet cell concentration in duplicate was determined by the method of Mills and Lee [15].

Sample Preparation for ELISA

The concentrations of intracellular and extracellular HC IgG in daily-taken samples were determined in duplicate by ELISA. The supernatant obtained by centrifuging as described above was kept frozen at -80°C until the ELISA analysis of extracellular HC IgG. The intracellular sample for intracellular HC IgG was retrieved by breaking cell walls as follows. Preweighed cell pellets were resuspended in 0.5 ml of high-salt buffer (HSB) solution of 50 mM Tris-HCl, 0.5 M NaCl, and 0.05% Nonidet P-40. Eighteen microliters of phenylmethyl sulfonylfluoride (PMSF) solution in isopropyl alcohol were added to HSB solution as a protease inhibitor. The cells suspended in the HSB/PMSF solution were sonicated in an ice water bath three times for 8 sec each. Then, the sample was centrifuged at 15,000 ×g for 5 min. The collected supernatant, accounting for the intracellular fraction, was stored at -80°C until the ELISA analysis of intracellular HC IgG.

ELISA Procedures

Capture ELISA [9] using anti-IgG antibody was used for the ELISA procedures. A 96-well immunoplate (Nunc Inc., Naperville, IL, U.S.A.) was coated with goat affinity-purified antibody to mouse IgG (Organon Teknik Corporation, Durham, NC, U.S.A.). The plate loaded with a sample was covered, and then placed in a 30°C-incubator for reaction. The coating antibody was allowed to bind to the immunoplate for 1 h, and then was washed with deionized (DI)-water three times. To prevent nonspecific binding of sample IgG or enzyme-conjugated antibody to noncoated area of the plate, blocking was done on the plate surface with a blocker solution containing 3% powdered milk, 0.5% gelatin, and 0.05% Tween-20 in phosphate buffer solution (3.8 mM NaH_2PO_4, 16.2 mM Na_2HPO_4, and 150 mM NaCl, pH 7.0). Fifty microliters of sample or standard IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.) were loaded onto the plate, and diluted with the blocker. The plate was incubated at 30°C for 2 h, and then rinsed with DI-water to remove any unbound antibody. For the detection of intracellular HC IgG, alkaline phosphatase conjugated with the goat affinity-purified antibody to mouse IgG was used. For the analysis of extracellular samples having low concentrations of IgG, goat anti-mouse IgG-alkaline phosphatase human adsorbed (Southern Biotechnology Associates Inc., Birmingham, UT, U.S.A.) was used. For binding of enzyme-conjugated antibodies to samples, incubation was carried out for 1 h. Then, the plate was rinsed with DI-water. Alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in the substrate solution (97.1 ml of diethanolamine and 50 ml of 1 M $MgCl_2$ in 100 ml DI-water, pH 9.8), and added to the well. After incubating the enzyme reaction mixture at 30°C for 30 min, the absorbance at 405 nm was measured on an ELISA reader (Bio-Tek Instrument Inc., Winooski, VT, U.S.A.).

Addition of Cytokinins

Four types of cytokinins were used in this study. Kinetin, IPA, BA, and zeatin were obtained from Sigma Chemical

Co. Cytokinins were dissolved in a small volume of 0.1 N NaOH and diluted with DI-water. Each flask contained different cytokinins of varying final concentrations, such as none (control), 0.02, 0.2, and 2 mg/l in each 60-ml modified MS medium. To study the effects of cytokinin in the presence of 2,4-D, each cytokinin together with 2,4-D were added to the media. The concentrations of cytokinins were varied in the same manner as the cytokinin study without 2,4-D. Cells were cultivated under the same conditions used for the stock suspension culture, as described earlier.

Addition of Organic Nitrogen Sources

To find the effects of various organic nitrogen sources on the cell growth and HC IgG production, each nitrogen source was added, to the modified MS medium containing 2,4-D at 0.2 mg/l, by varying concentrations such as none (control), 0.1, 0.5, 1, and 2 g/l. The suspension cultures were carried out under the same conditions as used for the stock suspension cultures.

Results and Discussion

Effects of Cytokinins Without 2,4-D

Figure 1 shows the typical cell growth and HC IgG production (intracellular and extracellular) in the suspension culture of the transgenic *N. tabacum*. As shown in Fig. 1, it appears that HC IgG production was strongly associated with cell growth. Figure 2 presents the productivity of cell mass (based on wet weight) obtained from a batch culture of transformed tobacco cells cultured for 7 days. The addition of kinetin as a growth regulator in place of 2,4-D resulted in poor cell growth. In particular, the doubling time of the cells with 2 mg/l kinetin was as long as 3.8 days in the exponential phase, whereas it was only 1.5 days

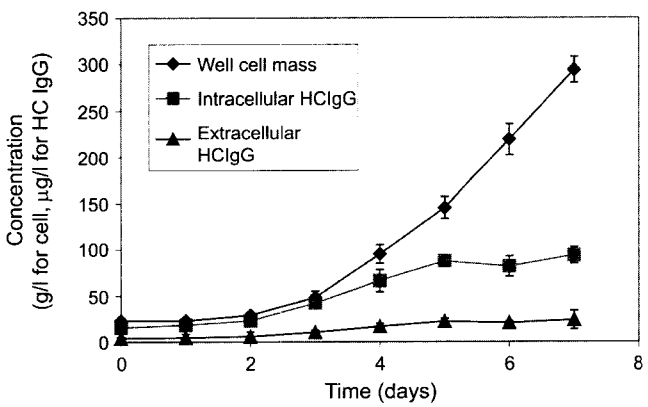

Fig. 1. A typical time course of cell growth and intracellular and extracellular HC IgG production by transgenic *N. tabacum*.
Cultivation was performed in the modified MS medium containing 2,4-D at 0.2 mg/l as a growth regulator. The error bars indicate the standard deviations from the means.

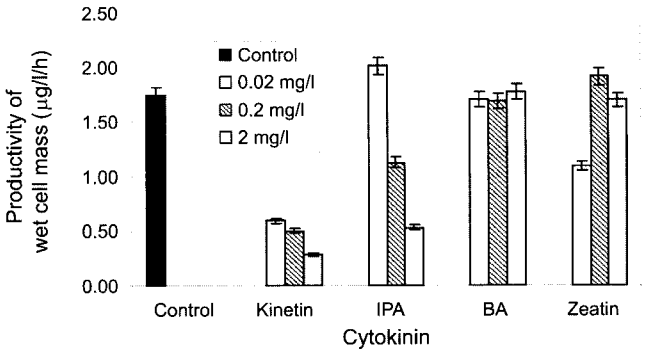

Fig. 2. Production of wet cell mass of transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the absence of 2,4-D.
Concentration of each cytokinin was 0.02, 0.2, and 2 mg/l in the modified MS medium without 2,4-D. The control experiment was carried out in the modified MS medium containing 2,4-D at 0.2 mg/l without any cytokinins. The error bars indicate the standard deviations from the means.

for the control. However, when IPA was added to the media at a final concentration of 0.02 mg/l, the production of wet cell mass was 2.01 g/l/h, which was a 15% increase from the control. The production of cell mass by the treatment of BA at all concentrations were very similar to those of the control. When BA was added at concentrations of 0.02 and 2 mg/l, the production of cell mass after 7 days culture were 1.70 and 1.77 g/l/h, respectively. The addition of zeatin at 0.2 and 2 mg/l gave similar production of wet cell mass.

Figures 3 and 4 demonstrate the production of both intracellular and extracellular HC IgG obtained in suspension cultures after 7 days culture, respectively. By comparing Figures 3 and 4, the production of extracellular HC IgG were much lower than those of intracellular HC IgG in all cases. This result indicates that most of the expressed HC

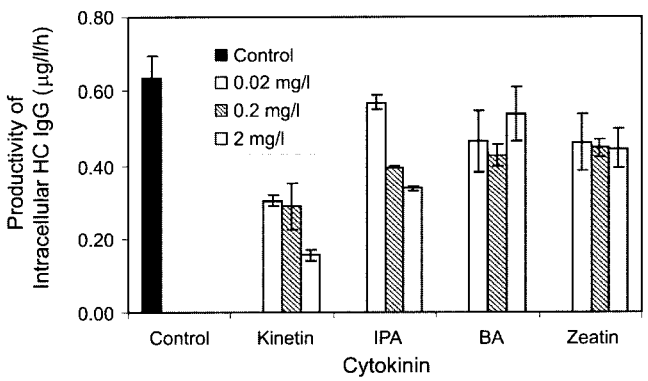

Fig. 3. Production of intracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the absence of 2,4-D.
Concentration of each cytokinin was 0.02, 0.2, and 2 mg/l in the modified MS medium without 2,4-D. The control experiment was carried out in the modified MS medium containing 2,4-D at 0.2 mg/l without any cytokinins. The error bars indicate the standard deviations from the means.

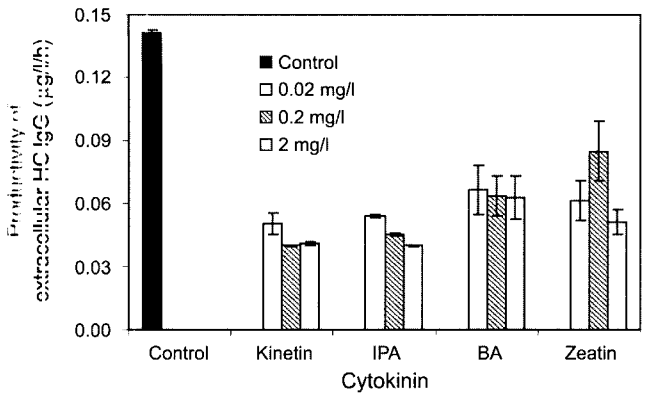

Fig. 4. Production of extracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the absence of 2,4-D.
Concentration of each cytokinin was 0.02, 0.2, and 2 mg/l in the modified MS medium without 2,4-D. The control experiment was carried out in the modified MS medium containing 2,4-D at 0.2 mg/l without any cytokinins. The error bars indicate the standard deviations from the means.

IgG remained inside the cells without being secreted. Also, the production of both intracellular and extracellular HC IgG were generally lower than the corresponding controls. This implies that all the cytokinins tested in this experiment were not effective for stimulating the HC IgG production by the transgenic tobacco cells. Among four different cytokinins, the production of HC IgG was most severely depressed when kinetin was added to the media. This negative kinetin effect is not in agreement with other earlier reports, in which the protein synthesis in plant cells was stimulated by kinetin [5, 16]. Since the stock cells used as inoculums in this study were maintained in the modified MS media containing 0.2 mg/l 2,4-D, one possible explanation for the negative response of *N. tabacum* to kinetin might have been that the sudden change in growth regulator from 2,4-D to cytokinins repressed the cell growth and foreign protein production.

Effects of Cytokinins Combined with 2,4-D

Since the addition of kinetin to the MS media without 2,4-D was not effective for promoting the IgG production, each cytokinin was added to the culture medium with 0.2 mg/l 2,4-D to reduce the possible negative effects by the abrupt change in growth regulator, as shown above. The concentrations of cytokinins were varied as none (control), 0.02, 0.2, and 2 mg/l.

In Fig. 5, the production of wet cell mass obtained by addition of different cytokinins combined with 2,4-D were compared with those of the control treated with 2,4-D only. The addition of 2,4-D with 0.2 mg/l of kinetin significantly increased the production of wet cell mass, from 0.5 g/l/h with kinetin only to 1.81 g/l/h with kinetin and 2,4-D. A similar result was obtained when IPA was added together with 2,4-D. However, when the concentration of cytokinin

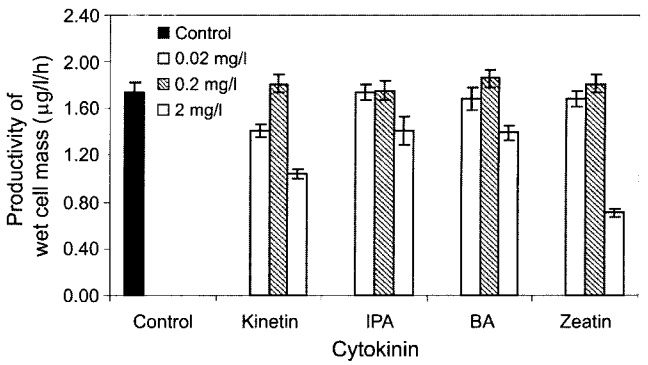

Fig. 5. Production of wet cell mass of transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the presence of 2,4-D.
Concentrations of each cytokinin were none (control), 0.02, 0.2, and 2 mg/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

was increased to 2 mg/l, it had adverse effects on cell growth, even in the presence of 2,4-D.

Figures 6 and 7 show the production of intracellular and extracellular HC IgG produced by *N. tabacum* cultivated under different cytokinins with 2,4-D for 7 days, respectively. As observed in the experiment without 2,4-D, the production of intracellular HC IgG were much higher than those of extracellular HC IgG. In Fig. 6, both kinetin and IPA at a final concentration of 2 mg/l in the media showed higher production of intracellular HC IgG, compared to the control, where the intracellular HC IgG production were 0.80 and 0.86 mg/l/h, respectively. The highest production of intracellular HC IgG (0.91 μg/l/h) was obtained, when BA was added at 0.2 mg/l, which was a 42% increase compared to the control. When zeatin was added at 0.2 mg/l with 2,4-D, the intracellular production of HC IgG was also higher in comparison with that of the control.

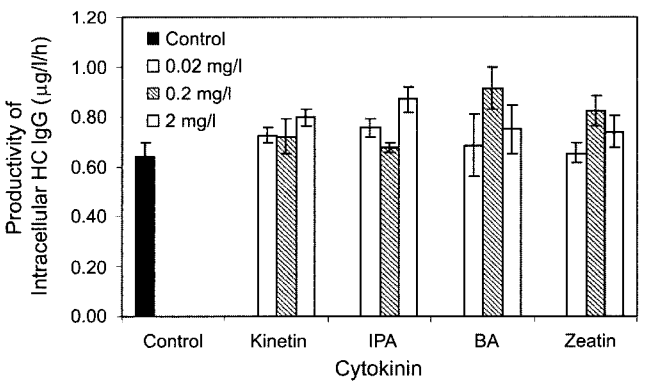

Fig. 6. Production of intracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the presence of 2,4-D.
Concentrations of each cytokinin were none (control), 0.02, 0.2, and 2 mg/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

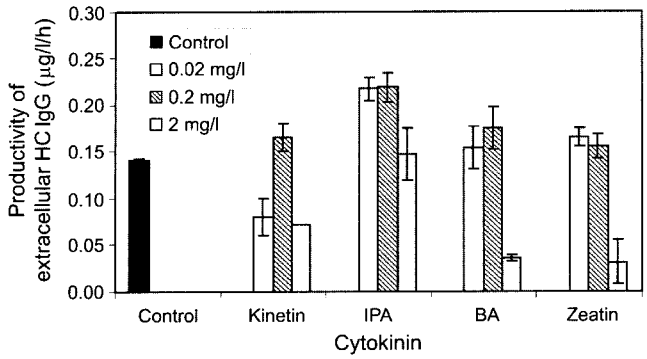

Fig. 7. Production of extracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days under various cytokinins including kinetin, IPA, BA, and zeatin in the presence of 2,4-D.
Concentrations of each cytokinin were none (control), 0.02, 0.2, and 2 mg/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

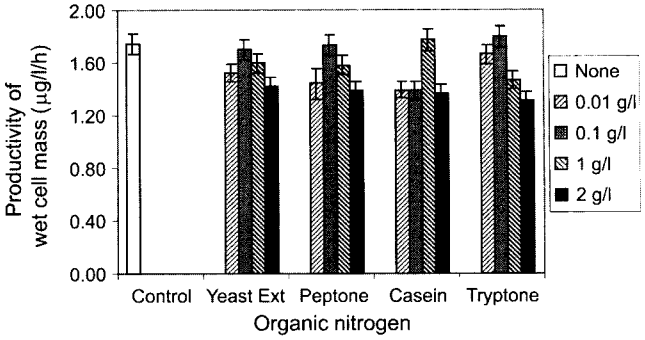

Fig. 8. Production of wet cell mass of transgenic *N. tabacum* cultivated for 7 days in the presence of various organic nitrogen sources including yeast extract, peptone, casein hydrolysate, and tryptone.
The concentrations of each nitrogen source were none (control), 0.01, 0.1, 1, and 2 g/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

The results shown in Fig. 7 demonstrate the stimulatory effect of cytokinin on the production of extracellular HC IgG in the presence of 2,4-D. With IPA added at a level of 0.2 mg/l, an increase of extracellular HC IgG by as much as 55% compared to the control was obtained. A similar effect on the extracellular HC IgG production was shown in the case of adding IPA at 0.02 mg/l. A lower concentration of BA and zeatin (0.02 mg/l) resulted in similar extracellular HC IgG production, compared to that of the control.

The enhanced production of IgG by adding cytokinins to the culture media with 2,4-D, which is opposite to the results obtained in the absence of 2,4-D, can be explained as follows. First, the addition of cytokinin with 2,4-D alleviated the possible negative effects due to sudden change of the growth regulator from 2,4-D to cytokinin. Second, cytokinins are known to be involved in the regulation of polyribosome formation [5], thus enhancing the initiation of protein synthesis. Cytokinins are also known to activate RNA-polymerase in plants by binding to a receptor in the cytoplasm, then penetrating the nucleus [13]. Therefore, it was concluded that the increased HC IgG production with the addition of cytokinin in the presence of 2,4-D was due to stimulation of protein synthesis by cytokinin, by minimizing the negative effects of the sudden change of growth regulator.

Effects of Organic Nitrogen Sources

The production of the transgenic tobacco cell mass obtained after 7 days under various organic nitrogen sources are shown in Fig. 8. Compared with the control without supplementation of any organic nitrogen sources, addition of organic nitrogen sources to the MS medium did not significantly affect the cell mass production. However, when the media were supplemented with a certain nitrogen source at specific concentrations (Fig. 9), the intracellular HC IgG production increased, compared to the control without any organic nitrogen sources. For example, adding casein hydrolysate at 0.01 g/l or tryptone at 1 g/l resulted in higher production of intracellular HC IgG than the control, where the production increased as much as 68 or 67% compared to the control. On the other hand, when yeast extract or peptone was added to the MS media, the intracellular HC IgG production was not significantly different from that of the control.

As seen in Fig. 10, it is noteworthy that the preferred nitrogen sources for the extracellular HC IgG production differed from those for the intracellular HC IgG production. Peptone was the most effective among the four nitrogen sources, followed by yeast extract. Yeast extract at 0.1 g/l also gave higher extracellular HC IgG concentrations compared to the control. Organic nitrogen sources including protein hydrolysates are reported to be good suppliers of amino acid [4, 8, 29] for plant cells and tissues capable of metabolizing various nitrogen sources [23, 26]. Therefore,

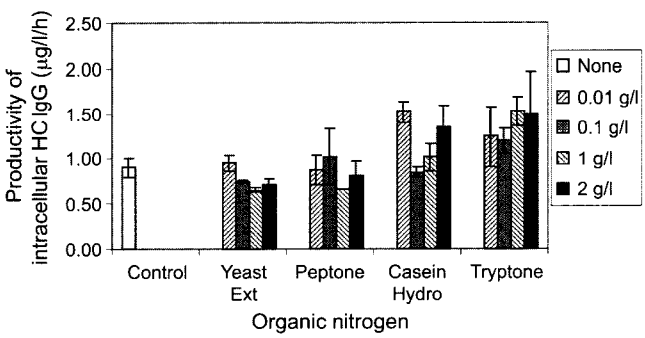

Fig. 9. Production of intracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days in the presence of various organic nitrogen sources including yeast extract, peptone, casein hydrolysate, and tryptone.
The concentrations of each nitrogen source were none (control), 0.01, 0.1, 1, and 2 g/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

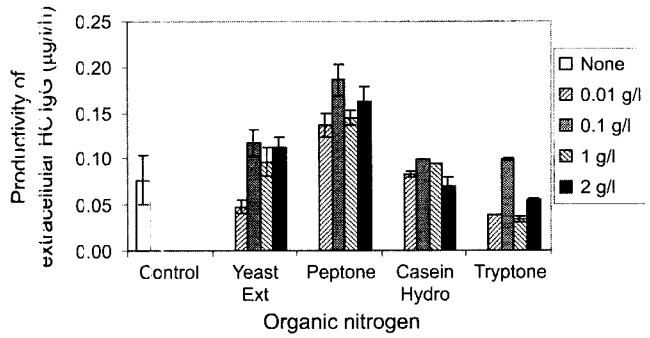

Fig. 10. Production of extracellular HC IgG by transgenic *N. tabacum* cultivated for 7 days in the presence of various organic nitrogen sources including yeast extract, peptone, casein hydrolysate, and tryptone.

The concentrations of each nitrogen source were none (control), 0.01, 0.1, 1, and 2 g/l in the modified MS medium containing 2,4-D at 0.2 mg/l. The error bars indicate the standard deviations from the means.

the supplement of the MS media with an organic nitrogen source is considered to significantly improve the foreign protein production in the cell cultures of a transgenic plant.

CONCLUSION

In the suspension cultures of transgenic tobacco cells, among four different cytokinins (kinetin, IPA, BA, and zeatin) added to the MS media, BA at a final concentration of 0.2 mg/l in the media in the presence of 2,4-D at 0.2 mg/l was the most effective for increasing the productivity of intracellular HC IgG by 43%, compared to the control with 2,4-D only. The addition of casein hydrolysate (0.01 g/l) or tryptone (1 g/l) to the MS media as an organic nitrogen source increased the intracellular production of HC IgG by 68 and 67%, respectively. Therefore, it was concluded that the HC IgG production could be significantly enhanced by optimizing the composition of media containing growth regulators and organic nitrogen sources.

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