【論　　文】

Effects of a Fermented Hot-Water Extract of *Stevia rebaudiana* Bertoni on Rooting under *in vitro* Stress Conditions

ステビア熱水抽出発酵液のストレス条件下での発根促進効果

岡　本　啓　湖

Keiko Okamoto*, Sumika Yamaguchi**, Naohiko Sato***

*:Department of Food and Fermentation Science Faculty of Food and Nutrition, Beppu University 82, Kita-ishigaki, Beppu,Oita,874-8501,Japan

**: Department of Applied Microbiology, Fukuoka Junior College of Agriculture, 250 Osano, Dazaifu-city, Fukuoka 818-0134, Japan

***: B&L Ltd., 3-16, Kandanishikichou, Chiyodaku, Tokyo, 101-0054, Japan

Key words: *Stevia rebaudiana* Bertoni, fermented hot-water extract, *Lactuca sativa* L, low-light illumination, IAA

ストレス条件下でステビア熱水抽出発酵液のレタス種子に対する発根・発芽に与える効果を検討した。その結果、古いグレートレーク種子の発根・発芽率が２倍に増加した。発根効果を担う物質を検討する為に、発根力の高い種子からの子葉をインドール酢酸（IAA）添加同実験系に供し子葉からの発根率を調べた結果、ステビア熱水抽出発酵液は低濃度 IAA では発根促進効果を示した。ステビア熱水抽出発酵液には IAA 促進物質の存在が示唆された。

Abstract

The effects of a fermented hot-water extract (JBB Stevia Laboratory) on the rooting and germination of lettuce seeds were investigated under two stress conditions (high seed age and low-light illumination), while the effects of the extract on growth were assessed under one stress condition (low-light illumination). The results showed that, when sterilized 11-month-old *Lactuca sativa* L (lettuce) seeds with rooting and germi-nation rates of 70% were incubated under low-light illumination of 600 lux, the rooting and germination rate of seeds incubated in agar media of pH 4.68 containing fermented hot-water extract were both 143% that of seeds incubated in the control media (plain agar media). Also, when using 12-month-old *L. sativa* seeds with a rooting and germi-nation rate of 50% under low-light illumination of 600 lux, the rooting and germination rate of seeds incubated in agar media of adjusted pH 5.6 containing 10

ppm of ferm-ented hot-water extract were 200 and 180% that of seeds incubated in the control media, respectively. In order to identify substances responsible for the promotion of rooting, *L. sativa* 366 seeds with high rooting viability were incubated and the resulting cotyledons were then incubated with different concentrations of indoleacetic acid (IAA) so as to investigate the effects of the extract on rooting. The results showed that the fermented hot-water extract with a low concentration of IAA (0.01 ppm) promoted rooting within the shortest time but with a high concentration of IAA (1.0 ppm) suppressed rooting. Also, by incubating seven-month-old *L. sativa* seeds with a rooting and germination rate of 100%, the effects of the fermented hot-water extract on growth after germination were examined under low-light illumination. The results showed that the fermented hot-water extract increased growth rate by 66.7% when compared to the control. Consequently, the components of the fermented hot-water extract were analyzed, and the results revealed that the nitrate and ammonia nitrogen contents of fermented hot-water extract media were only 0.00073 and 0.0013% those of Murashige-Skoog media.

Introduction

The plant, *Stevia rebaudiana* Bertoni is an economically valuable source of sweetening agents because it contains ent-kaurene diterpenoid glycosides, such as stevioside and rebaudioside (Farnworth, 1996). These sweetening agents have been the focus of int-ense research by the Japanese food industry (Abe and Sonobe, 1977; Morita, 1977; Okazaki et al., 1977; Tanaka, 1987; Yosihira et al., 1987). More than 100 compounds from the plant have been identified. In addition to diterpenoids, there are reports of the presence of other compounds including triterpenoids, sterols (Sholichin et al., 1980; Yasukawa et al., 1993), flavonoids (Rajbhandari and Roberts, 1983), volatile oils, and common phytochemicals (Fijita et al., 1977; Martelli et al., 1985; Cheng and Chang, 1983). Furthermore, the biochemical properties of these compounds have been actively researched, especially those of diterpene glycosides, which have been subjected to intense pharmacological and toxicological scrutiny (Pezzuto, 1986; Crammer and Ikan, 1987; Phillips, 1987; Kinghorn and Soejarto, 1991; Hanson and De Oliveira, 1993). After the sweetening agent extraction process, the leaves and discarded stems still contain tri-terpenoids, sterols, and flavonoids, and other parts of the plant contain volatile oils and common phytochemicals. These compounds, though valuable, have not been as thorou-ghly biochemically characterized as the sweetening agents. As a result, interest shown in these compounds has been high. As research on compounds other than diterpene glycol-sides has progressed, investigations into the use of *S. rebaudiana* Bertoni-derived products in several research fields have been conducted (JBB Stevia Laboratory, Saitama, Japan). In marine research, an extract from the stem of *S. rebaudiana* Bertoni is known to act as a potent antioxidant against *Sardinops melanostictus* oil and linolic acid (Xi et al., 1998), and a fraction possessing this high antioxidation activity contains high con-centrations of potassium (Xi et al., 1998). Also, in medical research, a fermented hot-water extract of this plant was found to possess an antibacterial activity against food pa-thogens, including Escherichia. coli O157 (Tomita et al., 1997). Furthermore in agricultural research, it was reported that *S. rebaudiana* extract completely cured pear tree with Monpa disease (*Rosellinia necatrix* infection) and accelerated rooting ability of that plant in the private farm (Kagoshima Prefecture, unpublished data). This demon-strated that

S. rebaudiana extract (fermented hot-water extract) shows potent antibacterial and antifungal activity against damaged or diseased plants, and can be used as an effective agricultural material. In the present study, we targeted seeds under stress conditions, such as high seed-age and low light illumination, investigated the effect of *S. rebaudiana* extract on rooting and germination abilities, and determined the growth of seeds under different stress conditions.

Materials and Methods

1. Effects on the rooting, germination, and growth of seeds

Seeds: 7-, 10-, 11-, and 12-month-old seeds of Lettuce, *Lactuca sativa* L (Great Lake 366), were prepared and stored at room temperature. Before germination, they were sterilized in 70% ethanol for 10 seconds, thoroughly washed using sterile water, sterilized in a sodium hypochlorite solution (available chlorine concentration 1%), then thoroughly rewashed using sterile water, and placed in culture media (10 seeds per medium). Rooting and germination were assessed after 7 d incubation and 30 d incubation, respectively. Growth was estimated by counting the number of seeds with the first foliage leaf.

Seed culture media: Plain agar media (pH 5.6) was prepared by dissolving 0.8% agar using distilled water, and Murashige-Skoog (MS) media (sucrose not added) was prepared by dissolving agar (0.8%) in a MS solution (pH 5.6). Fermented hot-water extract media was prepared by diluting the fermented hot-water extract to a concentration of 10, 100, or 1000 ppm. Diluted fermented hot-water extract was adjusted to pH 5.6 except for the experiment using 11-month-old seed. All culture media were sterilized at 121 ℃ for 20 minutes.

Seed incubation: During rooting assessment, seeds were incubated under low illumination conditions of 600 lux, and during growth assessment, seeds were incubated under illumination conditions of either 600 or 1500 lux. The incubation temperature was set at 30 ℃ for both assessments.

2. Effects on rooting of cotyledons

Cotyledons: *L. sativa* 366 seeds were germinated in plain agar media to obtain cotyl-edons.

Cotyledon culture conditions: The cotyledons were grown in either MS media (control) or 10-ppm fermented hot-water extract media. Indoleacetic acid (IAA) adjusted to 0.01, 0.1, or 1.0 ppm final concentration was added to the media. The cotyledons were grown under illumination of either 2000 or 2700 lux, at an incubation temperature of 30 ℃ for 20 d.

3. Confirmation of IAAby HPLC

The presence or absence of IAA was confirmed by liquid chromatography using the ethanol fraction of the fermented hot-water extract. Reversed-phase HPLC was performed using a 250 x 4.6 mm column (5 μ m) (Mightysil RP-18, Kanto Chemi-cal, Tokyo, Japan). A water-methanol gradient (0-80%) with a flow rate of 1 ml/min at 40 ℃ was used. A diode array (SPB-M 10A, Shimadzu Co., Japan) was used as a detec-tor.

4. Component analysis of the fermented hot-water extract

The phenol sulfate method was used for all saccharide analyses, while the brucine and indophenol methods were used to quantify nitrate nitrogen and ammonia nitrogen, respectively.

Results

1. Effects on the rooting, germination and growth of seeds

Figure 1 shows the number of *L. sativa* seeds that rooted in plain agar media over time; seeds used were 7, 10, 11, and 12 months old. After 7 d incubation, the rooting rate of 7-month-old seeds was high at 90%, thus demonstrating a high rooting viability. Although the rooting rate of 10-month-old seeds was also high at 90%, there was an observed lag in rooting time by 50%. The rooting rates of 11- and 12-month old seeds were 70 and 50%, respectively. Briefly, the older the seed used, the lower the rooting rate and the greater the time lag in rooting observed.

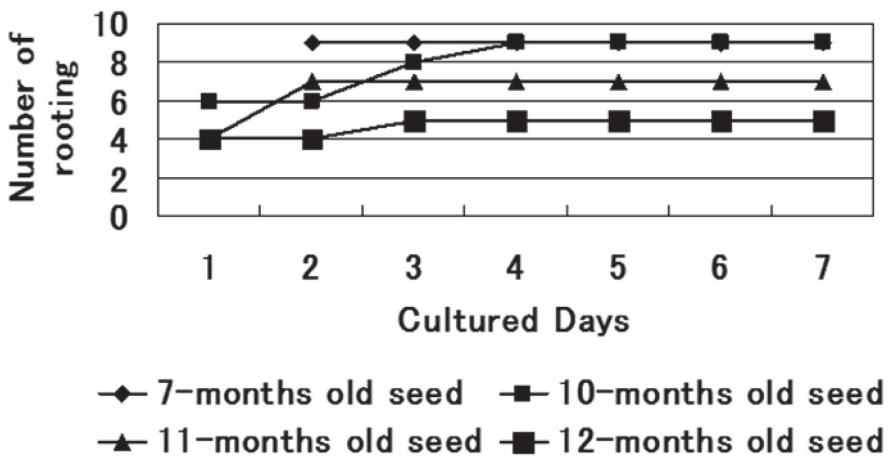

Fig.1 Effects of seed age using *Lactuca sativa* L.on the number of rooting in the agar-medium.

Figure 2 shows the number of *L. sativa* seeds that had germinated in plain agar media over time. After 7 d incubation, the germination rate of 7-month-old seeds was high at 90%, thus demonstrating high germination viability. Although the germination rate of 10-month-old seeds was also high at 90%, there was an observed lag in germination time by 75%. The germination rates of 11- and 12-month-old seeds were 70 and 50%, respectively. These findings show that the germination rate decreases and the lag-time of germination increases as the age of the seed increases. After ten months, the rooting and germination rates were correlated, and seeds only germinated when they first form-ed a root.

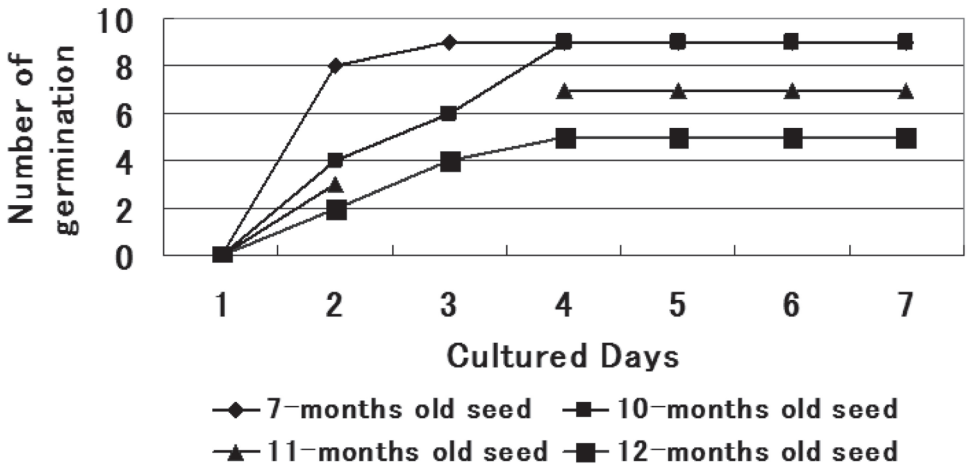

Fig.2 Effects of seed age using *Lactuca sativa* L.on the number of germination in the agar-medium.

Figure 3 shows the effect of low-pH fermented hot-water extract media on 11-month-old seeds with a rooting rate of 70% in plain agar media (control media: pH 5.6). The rooting rate of the 1000 ppm fermented hot-water extract media (pH 4.68) increase-ed by 143% when compared to that of control media.

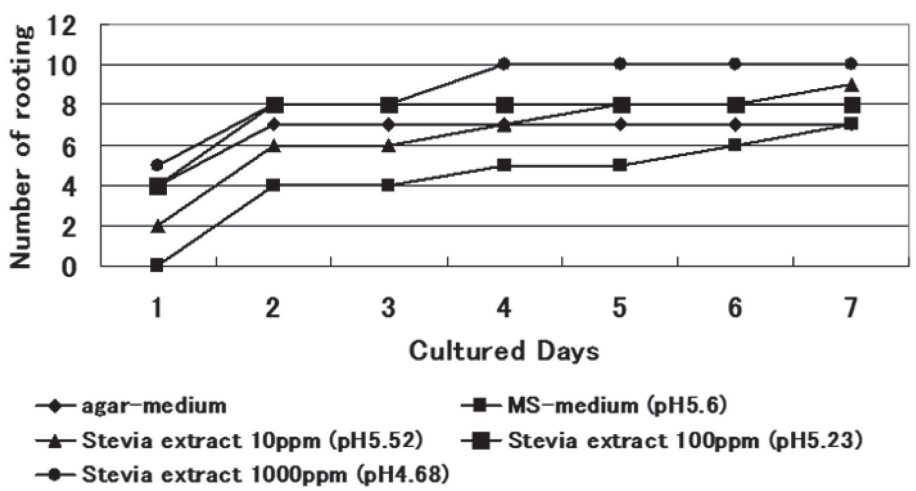

Fig.3 Effects of low pH in the fermented hot-water extract on the number of rooting
All measurements were performed with using the 11-months old seeds of *Lactuca sativa* L.

However, the rooting rate of the 100 ppm fermented hot-water extract media (pH 5.23) was 114% that of the control media (Figure 4).

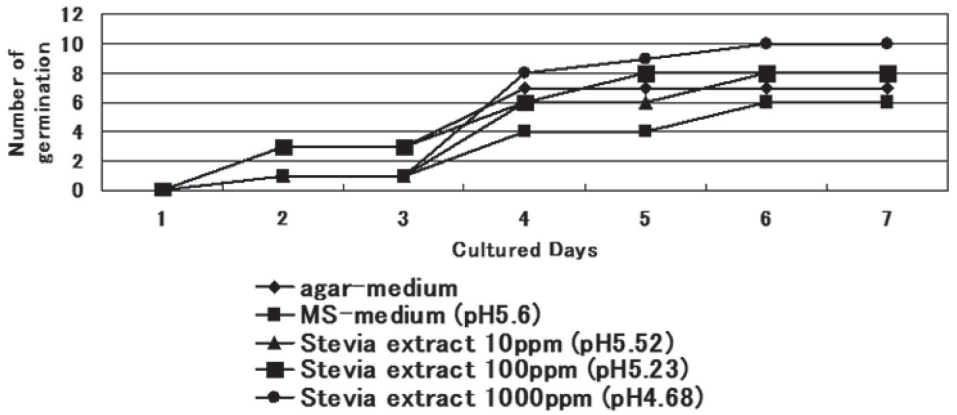

Fig.4 Effects of low pH in the fermented hot-water extract on the number of germination.
All measurements were performed with using the 11-months old seeds of *Lactuca sativa* L.

Furthermore, the correlation between germination and rooting rates observed in plain agar media was also present when fermented hot-water extract media was used, except for the 10 ppm fermented hot-water extract media (pH 5.52). To further investigate the effects of the fermented hot-water extract on rooting, the extract was diluted while ad-justing the pH of the culture media to pH 5.6 (Figure 5).

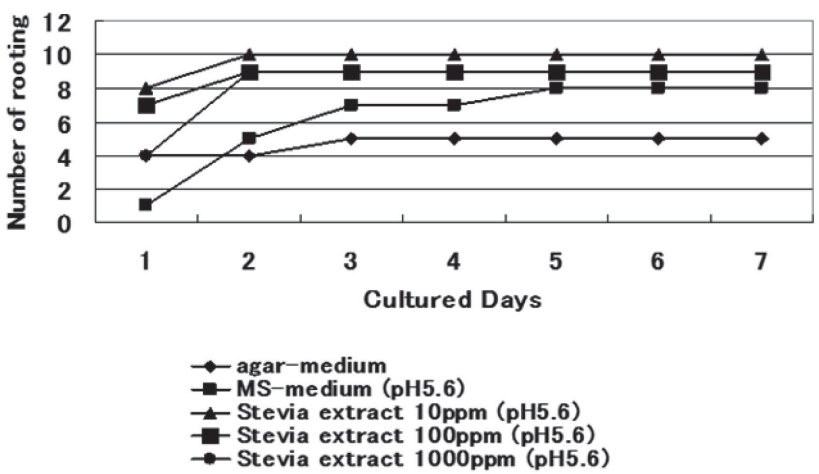

Fig.5 Effects of the fermented hot-water extract adjusted at pH5.6 on the number of rooting.
All measurements were performed with using the 12-months old seeds of *Lactuca sativa* L.

The rooting rate of the 12-month-old seeds in plain agar media was 50% that of the control, but the rooting rate of the 12-month-old seeds in 10, 100, and 1000 ppm fermented hot-water extract media was 200, 180 and 180% of the control, respectively. These findings suggest that the addition of fermented hot-water extract enhances the rooting rate by not only lowering

the pH, but also incorporating an as yet unknown rooting promotion agent. The extract with 10 ppm fermented hot-water extract media had the maximum rate of rooting promotion. The exact same results were observed when germination rates were analyzed (Figure 6), thus indicating that accelerated rooting facilitated germination.

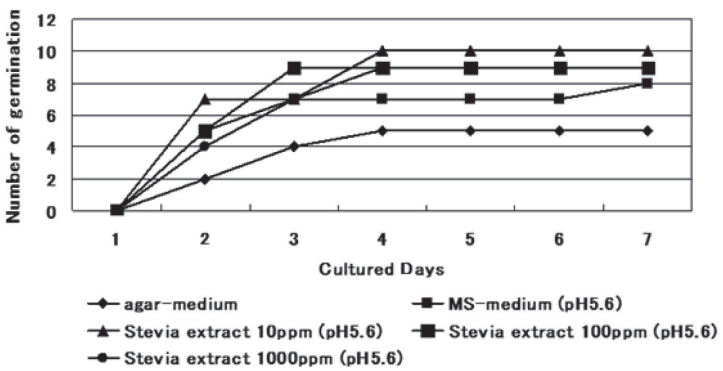

Fig.6 Effects of the fermented hot-water extract adjusted at pH5.6 on the number of germination. All measurements were performed with using the 12-months old seeds of *Lactuca sativa* L.

The effects of the fermented hot-water extract on the growth of seeds with a high rooting rate were investigated. Figure 7 shows the results of a growth experiment where 7-month-old *L. sativa* seeds (rooting rate: 100%) were incubated with an illumination of 600 lux. When compared to MS media control (pH 5.6), the growth rate of *L. sativa* was 66.7, 55.6, and 44.4% higher in the pH-adjusted (pH 5.6) fermented hot-water extract media, at concentrations of 100, 10, and 1000 ppm, respectively. The highest effect on the growth was given by 100ppm.

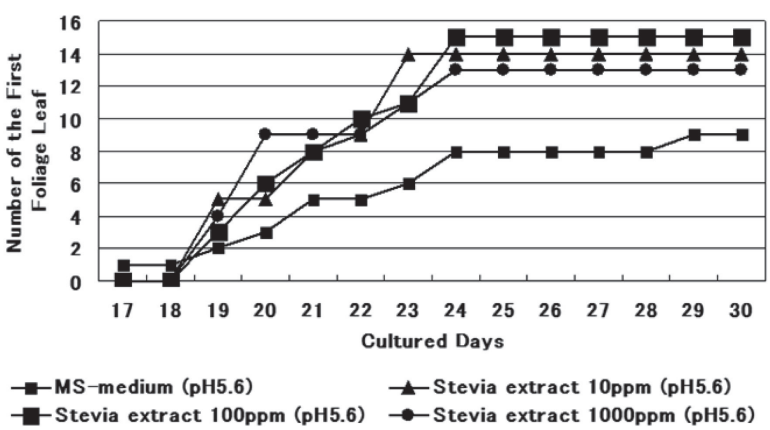

Fig.7 Effects of the fermented hot-water extract on the number of the first foliage leaf. All measurements were performed with using *Lactuca sativa* L.366 under the low-illumination (600lux). .Each medium was adjusted at pH5.6.

Concerning the growth rate of *L. sativa* compared to MS medium, 10ppm fermented hot-water extract medium increased by 5.3%, 100ppm fermented hot-water extract medium decreased by 5.3% and 1000ppm fermented hot-water extract medium was same when the illumination was increased to 1,500 lux. In this case, pH of each medium but MS medium was not adjusted (pH 5.6). These results derived that the fermented hot-water extract gave *L. sativa* the nutriments to grow under low illumination, or no effects of the fermented hot-water extract manifested under high illumination.

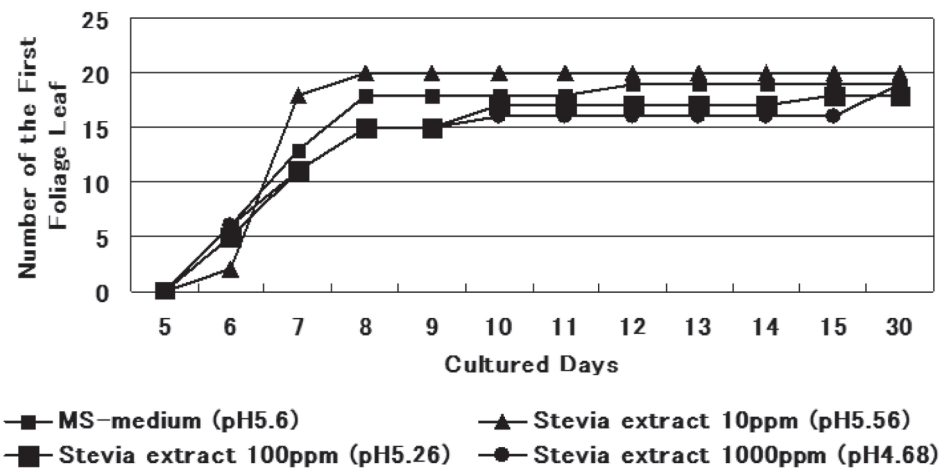

Fig.8 Effects of the fermented hot-water extract on the number of the first foliage leaf.
All measurements were performed with using *Lactuca sativa* L.366 under the high -illumination (1500lux). The pH of each medium was not adjusted to pH5.6.

These findings suggest that, under low illumination conditions where light levels are insufficient for post-germination photosynthesis, the fermented hot-water extract provided the nutrients necessary for growth. However, when cotyledons were photosynthetically self-sufficient, the addition of fermented hot-water extract to the media had a negligible effect on growth rate.

2. Effects on rooting of cotyledons

In order to investigate the relationship between rooting promotion and auxin-like hormone activity, cotyledons of *L. sativa* 366 were incubated in fermented hot-water extract media, and for comparison in MS media (control). Under 2000 lux of illumination, the rooting rate and speed of rooting in the 10 ppm fermented hot-water extract media were 250 and 185% greater than the control media, respectively. In addition, under 2700 lux of illumination, the rooting rate and speed of rooting in the 10 ppm fermented hot-water extract media were 133 and 142% greater than the control media, respectively (Figure 9). These findings suggest the presence of low levels of auxin-like substances in the fermented hot-water extract. This is supported by the fact that low levels of auxin facilitate root growth (Morre and Bonner, 1965). The effectiveness of the fermented hot-water extract was low under high illumination because lettuce seeds are photosynthetic seeds.To confirm the presence of auxin-like substances in the fermented hot-

water ext-ract, the effects of the extract on exogenous auxin were investigated.

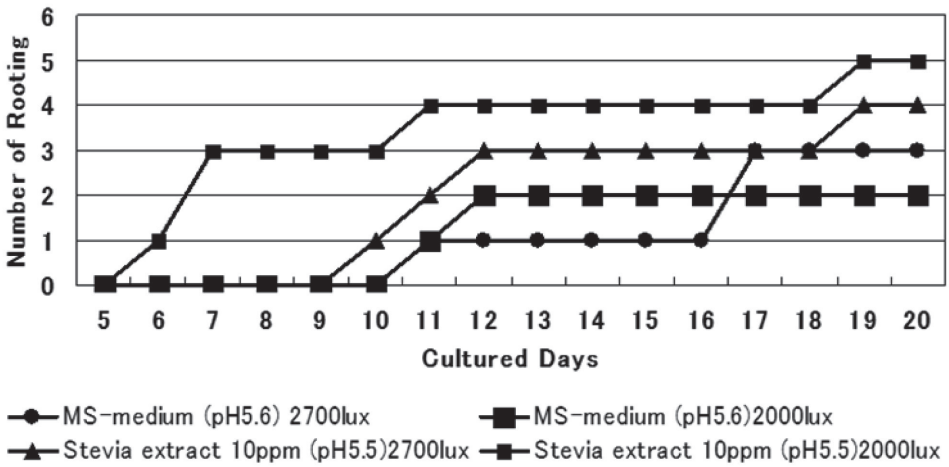

Fig.9 Effects of the fermented hot-water extract (Stevia extract) on the number of rooting.
All measurements were performed with using the cotyledos of *Lactuca sativa* L.366 at 2000lux for low-illumination or at 2700lux for high-illumination. The concentration of fermented hot-water extract was adjusted at 10ppm.

Figure 10 shows the effects of the extract on the rooting of cotyledons in media containing 0.01 ppm IAA. When compared to MS media containing 0.01 ppm IAA, grown under illumination of 2000 lux (control), the rooting rate and speed of rooting of cotyledons in the 10-ppm fermented hot-water extract media containing 0.01 ppm IAA increased by 350 and 296%, respectively. Furthermore, when the illumination was increased to 2700 lux, the rooting rate and speed of rooting of cotyledons in the 10-ppm fermented hot-water extract media containing 0.01 ppm IAA increased by 700 and 243% to those of the control media, respectively. However, under illumination of 2000 or 2700 lux, the rooting rate and speed of rooting of cotyledons in the 10-ppm fermented hot-water extract media containing 0.1 ppm IAA were 111 and 100% those of cotyledons in the MS media containing 0.1 ppm IAA, respectively (Figure 11).

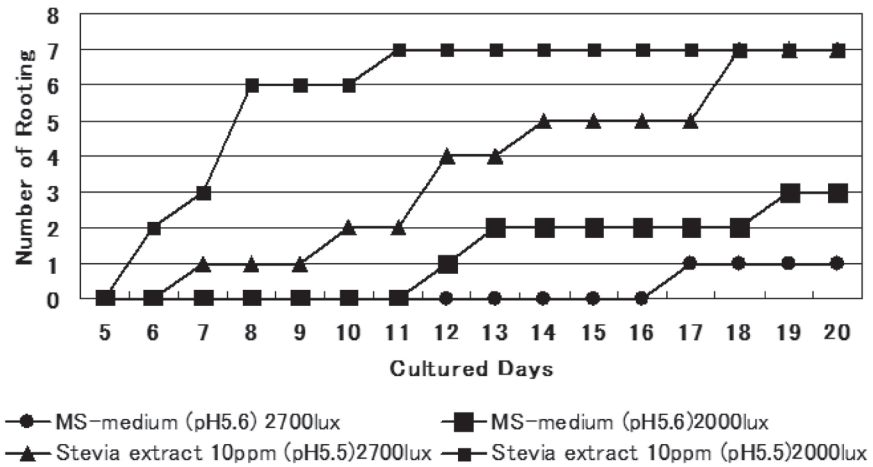

Fig.10 Effects of the fermented hot-water extract (Stevia extract) included 0.01ppm-IAA on the number of rooting. All measurements were performed with using the cotyledos of *Lactuca sativa* L.366 at 2000lux for low-illumination or at 2700lux for high-illumination. The concentration of fermented hot-water extract was adjusted at 10ppm.

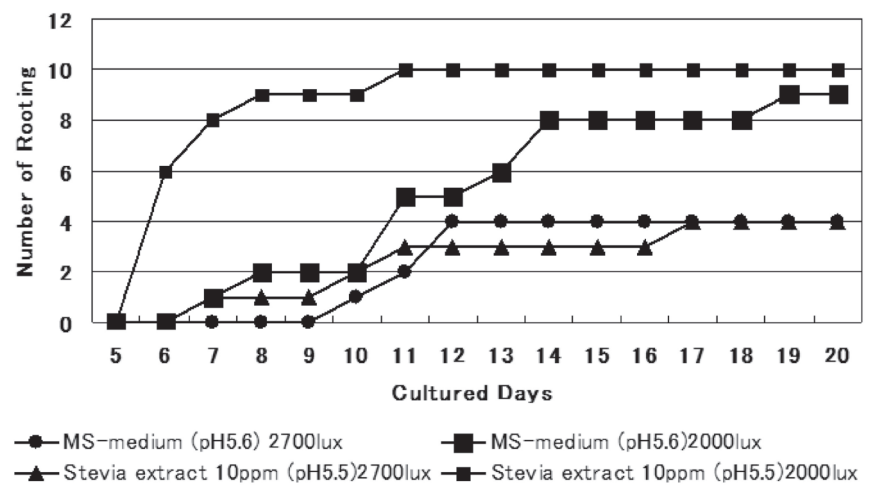

Fig.11 Effects of the fermented hot-water extract (Stevia extract) included 0.1ppm -IAA on the number of rooting. All measurements were performed with using the cotyledos of *Lactuca sativa* L.366 at 2000lux for low-illumination or at 2700lux for high-illumination. The concentration of fermented hot-water extract was adjusted at 10ppm.

Furthermore, under 2000 or 2700 lux of illumination, the rooting rate and speed of rooting of cotyledons in the 10-ppm fermented hot-water extract media containing 1.0 ppm IAA were 60 and 89% those of cotyledons in the MS media containing 1.0 ppm IAA, respectively (Figure 12).

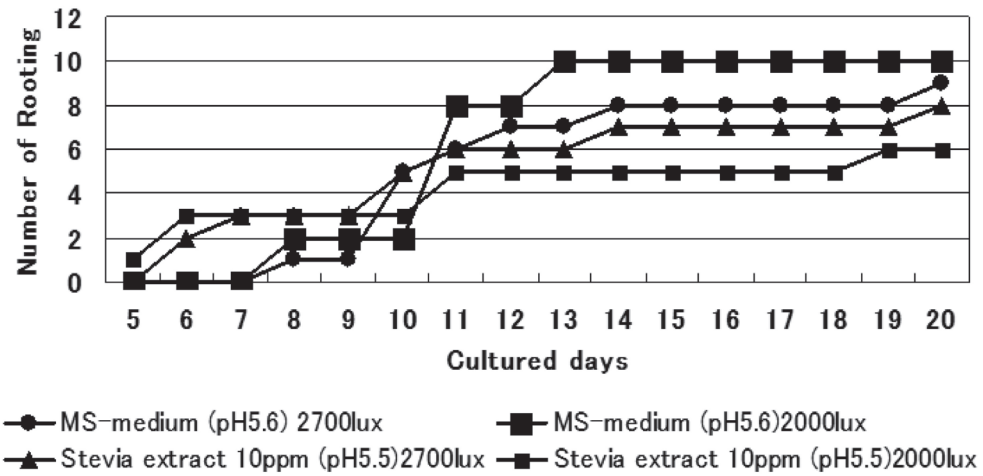

Fig.12 Effects of the fermented hot-water extract (Stevia extract) included 1.0ppm -IAA on the number of rooting. All measurements were performed with using the cotyledos of *Lactuca sativa* L.366 at 2000lux for low-illumination or at 2700lux for high-illumination. The concentration of fermented hot-water extract was adjusted at 10ppm.

These findings suggest that the promotive effects of the 10-ppm fermented hot-water extract media on rooting are at their maximum with 0.01 ppm IAA, and that the fermented hot-water extract suppresses rooting with 0.1 ppm or more IAA. In other words, the greater the IAA concentration, the greater the observed rooting suppression, thus further supporting the hypothesis that auxin-like substances are present in the fermented hot-water extract. In other words, a combination of auxin-like substances in the cotyledon, auxin-like substances in the fermented hot-water extract and exogenous IAA determined the promotion or suppression of rooting. This agrees with the finding that the presence of auxin above the optimal concentration inhibits root growth (Feldman, 1984).

3. Confirmation of IAA by HPLC

HPLC was used to confirm the presence auxin-like substances in the fermented hot-water extract. Compounds having the same UV spectra as the IAA standard were not found; however, compounds with peaks near the IAA standard were detected, thus suggesting the presence of auxin-related compounds, such as oxidized auxin (Lee et al., 1985), in the fermented hot-water extract. Therefore, it is believed that these compounds exhibited an auxin-like activity and worked in conjugation with endogenous auxin in the cotyledons.

4. Component analysis of the fermented hot-water extract

Table 1 shows the results of component analysis of the fermented hot-water extract. The levels of all saccharides (carbon source), and nitrate and ammonia (nitrogen source) were quantified. The results showed that the saccharide, nitrate nitrogen and ammonia nitrogen contents of the stock solution were 2.475 ± 0.0285%, 401 ± 4.67 ppm and 364 ± 17.0 ppm, respectively. The nitrate to ammonia nitrogen ratio was 1:1. The saccharide content of the 100 ppm fermented hot-water extract media was 0.2475 ± 0.00285 ppm. In addition, the nitrate and ammonia

nitrogen contents were only 0.00073 and 0.0013% those of the MS media.

Table 1 Concentration of elements included in the fermented hot-water extract

Element	Concentration (mg/ml)
Total Sugar	24.5 ± 0.29
Nitrate Nitrogen	0.401 ± 0.005
Ammonium Nitrogen	0.364 ± 0.017

Discussion

Drinkable fermented hot-water extract (Healthy Pocket, JBB Stevia Laboratory, Saitama, Japan), which is made mostly of the stem of *S. rebaudiana* Bertoni, has been reported to function as an antioxidant, exhibit antibacterial activity (in humans and animals), and prevent decay in fresh fish. A fermented hot-water extract made by an alternative process has been used in agriculture, and this extract was shown to be effective in treating pear trees infected with Mompa disease. Based on these findings, we investigated the effects of fermented hot-water extract on rooting, germination, and growth (first foliage leaf) of aged seeds. Since all the seeds were also photosynthetically competent, different levels of illumination were established. By varying these conditions, it was possible to change seed resistance against aging and attenuation of rooting ability under low illumination. The fermented hot-water extract is a product of fermentation and therefore has a low pH. The acidic conditions created by the addition of fermented hot-water extract to the media promoted rooting of L. sativa seeds under the experimental conditions used. Rapid root growth under acidic conditions (acid growth) has been documented by a number of researchers (Edwards and Scott, 1976; Tanimoto and Watanabe, 1986; Tanimoto et al., 1989). Furthermore, it has been suggested that acid growth is involved in the early stage of auxin-induced extension. However, our results show that rooting was promoted even in pH-adjusted fermented hot-water extract media, and thus suggests the presence of an auxin-like agent that functions with exogenous auxin. An explanation for the observed absence of IAA by HPLC could be that auxin-like substances underwent chemical changes caused by extremes in heating and lighting associated with the manufacture of the fermented hot-water extract. The fact that auxin enhances the stress resistance of plants (Mauk et al., 1987; Blanco-Brana et al., 1982; Hughes and Dickerson, 1990) indicate the existence of auxin-like substances in the fermented hot-water extract. It will be important to isolate and identify the compounds that promote rooting. Furthermore, the effects of the fermented hot-water extract on healthy seeds with normal rooting and germination activities can be ascertained by following the formation of the first foliage leaf under low light illumination. Although the nitrate nitrogen content (nitrogen source) of the fermented hot-water extract media was markedly smaller than that of the MS media, the fermented hot-water extract media were much more effective in promoting growth of the first foliage leaf. This difference in growth was attributed to factors other than differences in nitrogen content, specifically, the presence of a compound in the fermented hot-water extract. The saccharide content of the fermented hot-water extract was very low at 0.2475 ± 0.00285 ppm. Moreover, because

fermented hot-water extract media and MS media were comparable under high illumination (figure not shown), fermented hot-water extract media can be used as an alternative of MS media. One of the problems associated with large-scale plant incubation is the use of saccharides in culture media. The addition of saccharides in the media increases the risk of bacterial contamination. With fermented hot-water extract media, the risk of bacterial contamination should be greatly reduced.

Acknowledgements

We thank Mr. Yamada, assistant professor in the Pesticide Research Group of the Plant Protection and Epidemic Section, Material Development and Management Department, Kyushu University, for his help in auxin determination in the present study. The present study was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (14920038).

References

Abe, K. and Sonobe, M. 1977 Use of stevioside in the food industry; New. Food Industry, 19, 67-72

Blanco-Brana A., Jackson, J.E 1982 Effect of applying growth regulating hormones following fruit tree pruning: effects of different types and concentrations of auxin and GA4+7 and 6-benzylaminopurine on shoot emergence and wound healing of apple trees. J. Hort. Sci., 57, 17-30

Cheng, T.-F. and Chang, W.-H. 1983 Studies on the non-stevioside components of *Stevia* extract. National Science Cuncil Monthly, Taipei , 9, 775-782

Crammer, B. and Ikan, R. 1987 Progress in the chemistry and properties of the rebaudiosides. *In Developments in Sweetenerss-3*, T.H.Grenby(Eds), Elsevier Applied Science, London, pp 45-64

Edwards, K.I., and Scott, T.K 1976 Rapid-growth responses of corn root segments: effect of citrate-phosphate buffer on elongation. Planta, 129, 229-233

Farnsworth, N.R. 1996 NAPRALERT; A resource for research on medical plants. ICAST 96 Proceedings (The 12th International Conference on Advanced Science and Technology), University of Illinois at Chicago, Chicago, 6-8 April, pp 10-14

Feldman, LJ. 1984 Regulation of root development. Ann. Rev. Plant Physiol., 35, 223-242

Fujita, S.-I., Taka, K. and Fujita, Y. 1977 Miscellaneous contributions to the essential oils of plants from various territories. XLI. On the components of the essential oil of *Stevia rebaudiana* Bertoni, Yakugaku Zassi (in Japanese), 97, 692-694

Hanson, J.R. and De Oliveira, B.H. 1993 Serovioside and related sweet diterpenoid glycosides. Natural Products Reports, 10, 301-309

Hughes, R.K, and Dickerson, A.G. 1990 Auxin regulation of the response of *Phaseolus vulgaris* to a fungal elicitor. Plant Cell Physiol., 31, 667-675

Kinghorn, A.D. and Soejarto, D.D. 1991 Steviosidee. *In Alternative Sweetners* (2nd edn, revised and espanded), L.O'. Brien Nabors and R.C. Gelardi(Eds), Marcel Dekker, New York, pp 157-171

Lee, T.T., Starratt, A.N. and Jevnikar, J.J. 1985 Separation of conjugates and oxidative metabolites of indol-3-acetic acid by high-perfomance liquid chromatography. Journal of Chromatography, 325, 340-345

Martelli, A., Frattini, C. and Chialva, F. 1985 Unusual essential oils with aromatic properties., 1.Volatile components of Stevia rebaudiana Bertoni. Flavour and Fragrance Journal, 1, 3-7

Mauk, C.S., Bausher, M.G. and Yelenoski, G. 1987 Physiological effects of temperature and growth regulators on foliar chlorophyll, soluble protein and cold hardiness in citrus. Plant Growth Regul., 5, 141-154

Morre, D.J. and Bonner, J. 1965 A mechanical analysis of root growth. Physiol. Plant., 18, 635-649

Morita, E. 1977 Outlook for the Stevia natural sweetening agents. Syokuhin to Kagaku (in Japanese), 19, 83-87.

Okazaki, K., Nakayama, Y. and Owada, K. 1977 Stevia-new natural sweetener. Seikatsu Eisei (in Japanese), 21, 181-185

Pezzuto, J.M. 1986 Chemistry,metabolism and biological activity of steviol (ent-13-hydroxykaur-16-en-19-oic), the a glycine of stevioside. *In New Trends in Natyral Products Chemistry,* Elsevier Science, Atta-ur-Rahman and P.W. Le Quesne(Eds), Amsterdam, pp 371-386

Phllips, K.C. 1987 Stevia;Steps in developing a new sweetener, *In Developments in Sweeteners-3,* T.H. Grenby(Ed.), Elsevier Applied Science, London, pp 1-43

Rajbhandari, A. and Roberts, M.F. 1983 The flavonoids of *Stevia rebaudiana.* Journal of Natural Products, 46, 194-195

Sholichin, M., Yamasaki, K., Miyama, R., Yahara, S. and Tanaka, O. 1980 Labdane-type diterpenes from *Stevia rubaudiana*. Phytochemistry, 19, 326-327

Tanaka, O. 1987 Natural sweet principles: recent advance. Kagaku to Kogyo (in Japanese), Osaka, 61, 404-410[Chemical Abstracts 1987 107,233038m]

Tanimoto E., and Watanabe J. 1986 Automated recording of Lettuce root elongation as affected by auxin and acid pH in a new rhizometer with minimum mechanical contact to roots. Plant Cell Physiol., 27, 1475-1487

Tanimoto, E. et al 1989 Inhibition of acid-enhanced elongation of zea mays root segments by galactose. Plant Physiol , 90, 440-444

Tomita, T., Sato, N., Shiraishi, H., Sato, M., Takeichi, T. and Kamio,Y. 1997 Bacterial activity of a fermented hot-water extract from *Stevia Rebaudiana* Bertoni toward enterohemorrhagic Escherichia coli O157:H7 and other foodbron pathogenic bacteria. Microbio. Immunol., 41,(12), 1005-1009

Yasukawa, K., Yamaguchi, A., Arita, J., Sakurai, S., Ikeda, A. and Takido, M. 1993 Inhibitory effect of edible plant extracts on 12-o-tetradecanoyphorbol-13-acetate induced ear oedema in mice. Phytotherapy Reseach, 7, 185-189

Yosihira, K., Matsui, M. and Ishidate, M. 1987 Chemical characteristics and baiological safty[site] of a glycoside sweetener, stevioside. Tokishikoroji Foramu (in Japanese), 10, 281-289

Xi, YC., Yamaguchi, T., Sato, M. and Takeuchi, M. 1998 Antioxidant activity of *Stevia rebaudiana*. Journal of the Japanese Society for Food Science and Technology, 45(5), 310-316

Xi, Y.C., Yamaguchi, T., Sato, M. and Takeuchi, M. 1998 Antioxidant mechanism of *Stevia rebaudiana* extract and antioxidant activity of inorganic salts. Journal of the Japanese Society for Food Science and Technology, 45(5), 317-322