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Original Paper

Unique growth stage-dependent anti-inflammatory and immunostimulating effects of white bamboo (makomotake) on RAW264 macrophages shown by no production

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White bamboo is the swollen stem of *Zizania latifolia* parasitized by the smut fungus *Ustilago esculenta*. Five samples of galls were harvested at different stages of swelling, along with young seedlings of *Z. latifolia* and isolated colonies of *U. esculenta*. The inhibition capacity of boiling water or 50 % ethanol extracts on NO release by RAW264 cells, which was stimulated with lipopolysaccharide, was evaluated as anti-inflammatory activity, while the NO induction capacity was evaluated as immunostimulatory activity. Total polyphenol (TPP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity were also measured. The anti-inflammatory effect, as well as TPP and DPPH, was comprehensively detected in the ethanol extracts. The immunostimulating activity was observed in the boiled extracts at different levels, depending on the swelling stage, and was especially high in the top (apical) part. These data may indicate that functional components could be dynamically induced by interactions between *Z. latifolia* and *U. esculenta*.

Keywords: edible fungus, food function, RAW264 macrophages, *Zizania latifolia*, *Ustilago esculenta*

Introduction

The edible gall of *Zizania latifolia*, which is a stem swelling induced by the parasitic fungus *Ustilago esculenta*, is called "white bamboo", "*jiaobai*" or "*gausun*" in China (Chan and Thrower 1980, Chung and Tzeng 2004b) and "*makomotake*" in Japan (Kawagishi *et al.*, 2006; Tu *et al.*, 2019). As a well-liked vegetable with unique flavour and texture, white bamboo has traditionally been cultivated in East Asia (Guo *et al.*, 2007). Health-promoting properties of white bamboo were documented as early as 1596 in the historical book *Bencao Gangmu* (Compendium of Materia Medica) (Yaniv and Bachrach 2005), where claims, such as the revitalization of five organs and relief from a reddish face resulting from alcohol consumption, ulcerations and red eyes, are reported. Recent studies have also described some medicinal properties of white bamboo, including the prevention of hypertension and cardiovascular and hepatic diseases (You *et al.*, 2011).

Contrary to these rather empirical statements, the anti-osteoporosis effect of white bamboo was reported with identification of the responsible N-glycoside compounds makomotindoline (Kawagishi *et al.*, 2006) (Suzuki *et al.*, 2012) and makomotines (Choi *et al.*, 2014). More recently, water-soluble polysaccharides with beta-glycosidic linkages were isolated from white bamboo and showed specific immunostimulating activity on murine macrophage RAW264 cells, as well as antioxidant activity (Wang *et al.*, 2017; Wang *et al.*, 2018).

Mushroom-based (Yan *et al.*, 2018a; Mallard *et al.*, 2019) or algal (Kadam *et al.*, 2015) beta-glucans and gram-negative bacterial lipopolysaccharides (LPSs) (Zhu *et al.*, 2015) are well-known immunostimulatory polysaccharides that activate

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macrophages, which are expected to contribute to the enhancement of the body defence system against invasive pathogens (Kawai and Akira 2011). A typical and simple sign of macrophage activation is the secretion of nitric oxide (NO) by cells. In addition to their immunostimulatory effect, RAW264 macrophages were used to assess the anti-inflammatory activities of extracts based on their suppressive effects on NO secretion by LPS-stimulated cells (Wu *et al.*, 2017; Owolabi *et al.*, 2019; Mallard *et al.*, 2019) (Yamada *et al.*, 2019).

During gall swelling, the interaction between stem tissue of *Z. latifolia* and growing hyphae of *U. esculenta* is likely to contribute to the induction of functional components, including makomotines (Choi *et al.*, 2014) or unique polysaccharides (Wang *et al.*, 2018). We have previously reported that the interaction between *Z. latifolia* and *U. esculenta* influences microbial diversity, depending on the growth stages of the swollen stem, as well as on variable amounts of *U. esculenta* (Tu *et al.*, 2019). Herein, we focused on evaluating the anti-inflammatory and immunostimulating effects of white bamboo, depending on the stem enlargement process and in different parts harvested at optimal times.

Materials and Methods

Samples Fresh edible galls of white bamboo were harvested at Ito Noen, Komono-cho, Mie Prefecture (Tu *et al.*, 2019). Samples collected during different stages of swelling, from small (Nos. 1 and 2; tender) to large (No. 5; aged), were used, in which sample Nos. 3 and 4 (mature) were stems harvested at the optimal harvest times, and No. 5 contained many black spores of *U. esculenta*. The approximate length of each sample was 15 cm for No. 1, 20 cm for No. 2, 25 cm for No. 3 and 30 cm for Nos. 4 to 5. An individual edible gall collected at the optimal harvest stage was divided into 3 parts: top (apical side), middle (internode) and bottom (node). Additionally, seedlings (not swollen) of *Z. latifolia* and isolated fungal bodies of *U. esculenta* were also collected (Tu *et al.*, 2019).

Pretreatment All botanical samples were cut into pieces and dried under a stream of hot air (45 °C) and subsequently powdered by a pulverizer (Iwatani, Tokyo, Japan). *U. esculenta* isolated from white bamboo was cultured as hyphae in potato dextrose broth (Becton Dickinson and Company, NJNJ, USA) and collected by centrifugation as a wet fungal body.

Fifty percent ethanol extract Fifty percent ethanol was added to the powdered samples (100 mg mL^{-1}). The mixture was shaken vigorously for 10 min (SA-31, Yamato) and centrifuged at 16 200 g for 10 min at 25 °C. Aliquots of 1 mL of the supernatant were dispensed into 2-mL tubes and stored at –30 °C. The ethanolic extract was diluted 10- to 40-fold with culture medium for the cell-based assay, distilled water for the total polyphenol (TPP) assay and 50 % ethanol for the 2,2-diphenyl-1-1-picrylhydrazyl (DPPH) radical scavenging assay.

Boiled extract Distilled water was added to the powdered samples (100 mg mL^{-1}). The mixture was boiled (100 °C) for 10 min and then centrifuged at 16 200 g for 10 min at 25 °C. Aliquots of 1 mL of the supernatant were dispensed into 2-mL tubes and stored at –30 °C. The boiled extract was diluted 10- to 40-fold with culture medium for the cell-based assay.

Cell culture Mouse RAW264 cells (Riken BRC, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (FUJIFILM Wako, Osaka, Japan) supplemented with 10 % foetal bovine serum (Life Technologies, NY, USA) and a 1 % penicillin-streptomycin solution (FUJIFILM Wako) at 37 °C under 5 % CO_2.

Anti-inflammatory assay The inhibition capacity of NO release from RAW264 cells stimulated by LPS was evaluated as anti-inflammatory activity as follows (Yamada *et al.*, 2019). A 50-µL aliquot of a cell suspension (2×10^6 cells mL^{-1}) in culture medium was seeded into a well of a 96-well plate containing 10 µL of 1 µg mL^{-1} LPS (127-05141, FUJIFILM Wako) and 50 µL of a sample extract diluted with culture medium. The mixture was incubated for 24 h at 37 °C under 5 % CO_2. The supernatant medium (80 µL) was transferred to a new 96-well plate, and an equal volume of Griess reagent (equal volume mixture of 0.1 % *N*-(1-naphthyl) ethylenediamine dihydrochloride and 1 % sulfanilamide) was added. The concentration of NO released from activated RAW264 cells into the supernatant was measured based on the absorbance of nitrite, a product of NO produced by natural oxidation, for 10 min at 550 nm using an Emax microplate reader (Molecular Devices, CA, USA). The NO release of each sample was calculated as the relative percentage compared to the untreated control. Quercetin (Cayman Chemical, MI, USA) was used as a positive control of the inhibitory assay for NO production. The working 200 µmol L^{-1} solution was prepared by 100-fold volume dilution with medium at the time of use from the 20 mmol L^{-1} DMSO-ethanol (1:9) stock solution.

Immunostimulatory assay The induction capacity of NO release from RAW264 cells was evaluated as immunostimulatory activity. According to the same procedure as in the anti-inflammatory assay except for the removal of LPS stimulation, the mixture of the cells and sample extract was incubated, and the NO concentration in the supernatant was measured. The NO release of each sample was calculated as the relative percentage compared to the LPS-stimulated control. Fucoidan from *Fucus vesiculosus* (Sigma-Aldrich, MO, USA) was used as a positive control for the NO induction assay. The working 1 mg mL^{-1} solution was prepared by 10-fold volume dilution with medium at the time of use from the

10 mg mL^{-1} aqueous stock solution.

Cell viability assay The cell viability of each sample immediately after the removal of the supernatant as described above was measured based on the ability to convert a tetrazolium salt, WST-1 (Dojindo, Kumamoto, Japan), to a formazan dye. A 100-µL aliquot of the WST-1 reagent mixture (450 µmol L^{-1} WST-1, 18 µmol L^{-1} 1-methoxy-PMS (Dojindo), 0.9 × PBS) prepared immediately before use was added to the residual cells and incubated at 37 °C for 1 h. The absorbance of each sample well was measured at dual wavelengths of 450/650 nm, and the cell viability was calculated as the relative percentage compared to each control.

TPP assay Folin-Denis reagent (Sigma-Aldrich) was diluted 5-fold with distilled water just before use. Quercetin was used as a polyphenol standard. The prepared sample extracts were diluted 5-fold with distilled water. Aliquots of 50 µL of the diluted sample extracts were placed in a well of a 96-well microplate in duplicate and mixed with 50 µL of the diluted Folin-Denis reagent. A 100 µL aliquot of 4 % Na_2CO_3 was added and mixed well. After standing for 10 min at room temperature, the absorbance was measured at 650 nm with a microplate reader. For each sample, the corresponding absorbance of the sample blank in which the Folin-Denis reagent was replaced with distilled water was subtracted, and the final polyphenol concentration was obtained from the calibration curve as quercetin equivalents (mg-QE g^{-1}).

DPPH assay DPPH (FUJIFILM Wako) was freshly dissolved in 99.5 % ethanol to make a 1 mmol L^{-1} solution at the time of use. DPPH reactive reagent was prepared by mixing an equal volume of 0.3 mol L^{-1} MES buffer at pH 6.1 (Sigma-Aldrich) and 1 mmol L^{-1} DPPH solution. Aliquots of 50 µL of the sample solutions diluted with 50 % ethanol were added into wells of a 96-well microplate in duplicate and mixed with 100 µL of the DPPH reactive reagent. After standing for 10 min in the dark, the absorbance was measured at 550 nm with a microplate reader. The sample blank, in which the DPPH reactive reagent was replaced with distilled water, was subtracted, and the final DPPH radical scavenging activity was determined as Trolox equivalents (µmol-TE g^{-1}).

Statistical analysis The results of the cell-based assays are expressed as the mean values and standard deviations (SD), n=6. The TPP and DPPH assays were conducted in duplicate. One-way analysis of variance (ANOVA) followed by Tukey's test was adopted for statistical comparisons using GraphPad Prism 7 (GraphPad, CA, USA). Differences were deemed significant at $p < 0.05$.

Results

Anti-inflammatory effect The anti-inflammatory effect, evaluated as the inhibition of NO production, was comprehensively determined in the 50 % ethanol extracts (Fig. 1). Among the samples from different swelling stages, only white bamboo sample No. 2 (the second smallest sample) showed distinctive inhibition of NO production in both the boiled and the 50 % ethanol extracts. However, a sample collected at the optimal harvest time had a much higher inhibition rate, especially in the 50 % ethanol extract, regardless of the stem part (top, middle or bottom). The whole mixture and seedlings also showed relatively high inhibition of NO production. In contrast, the extracts of *U. esculenta* fungal bodies showed rather weak inhibition with a NO production rate of 46.1 % in the 50 % ethanol extract. Cell viability, except that observed with the divided stem parts, decreased to approximately 70 % or 40 % as a minimum for the seedlings and sample No. 2 (Fig. 1). These two samples that showed critical reductions of cell viability corresponded to the samples with distinctive anti-inflammatory activities, as described above. On the contrary, especially for the boiled extracts of divided stem parts, cell viability increased from 120 to 140 %, maintaining the anti-inflammatory tendency for the middle and bottom parts.

Immunostimulating effect The promotion of NO production, used to evaluate the immunostimulating effect, was specifically observed in boiled extracts with different intensities from the different swelling stages and parts of white bamboo (Fig. 2). Samples in the earliest swelling stage (No. 1) and the later stages (Nos. 4 and 5) showed high promotion of NO production. Among the divided parts from the sample collected at the optimal harvest time, only the top part showed exclusive NO promotion. In addition, almost no NO production was detected in other extracts, including those of the mixture, seedlings or *U. esculenta* extracted with either boiling water or 50 % ethanol. The influence on cell viability in this immunostimulation experiment showed the same tendency as the aforementioned anti-inflammatory effects. The decreases in cell viability were rather significant, by approximately 50 % or more, except with samples Nos. 1, 4, and 5 and the top part, which retained almost 100 % cell viability (Fig. 2). These four samples that did not reduce cell viability corresponded to the samples that showed immunostimulating activity, although systematic activation by the stem segments was not observed in this experiment.

The boiled extract of the top part of the sample, which showed the highest NO-promoting effect, was diluted 20- to 160-fold (5 to 0.625 mg mL^{-1}) and tested (Fig. 3). To evaluate the contribution of native LPSs to the promoting effect, LPSs were removed from the extract solution using a specific affinity column (EndotoxinOUT, FUJIFILM Wako). The column-treated eluate was diluted and measured as well. The NO-promoting activity was weakened but still maintained, and the dose dependency was clearly disclosed. The LPS removal treatment also led to a decrease in cell viability when treated

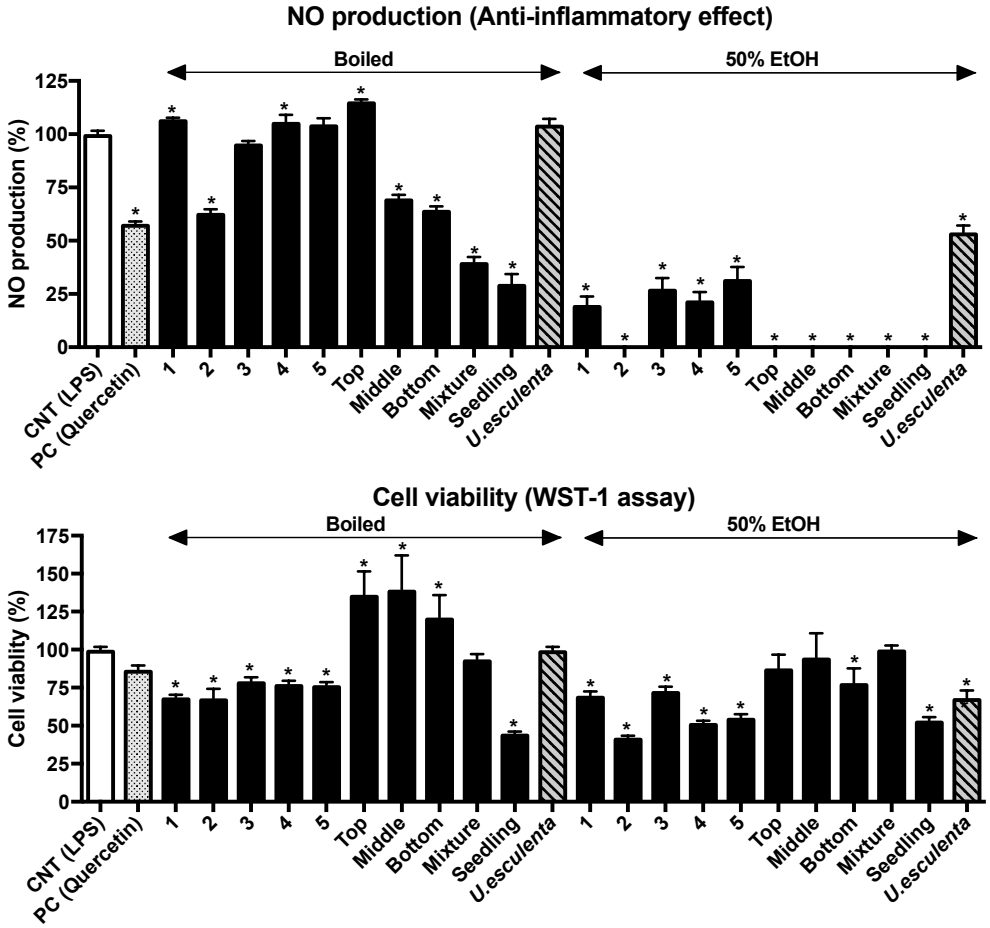

Fig. 1. Anti-inflammatory effects (upper) and effects on cell viability (lower) of white bamboo samples. Relative NO production and cell viability are shown for both boiled (left) and 50 % ethanol (right) extracts compared to the untreated control (cell medium). The data were obtained in the presence of LPSs. The numbers 1 to 5 correspond to the stage of swelling, from small to large. Another white bamboo sample collected at the optimal harvest time was divided into top (apical side), middle (internode) and bottom (node) segments. The "seedling" sample contained leaves of *Z. latifolia*. "*U. esculenta*" was a sample of fungal bodies. Samples were prepared at a concentration of 10 mg mL^{-1} by dilution of each extract with cell medium. Positive control (PC) was a quercetin solution at 200 μmol L^{-1}. Each column represents the mean + standard deviation, n = 6. Asterisks indicate significant differences compared to the control ($p < 0.05$).

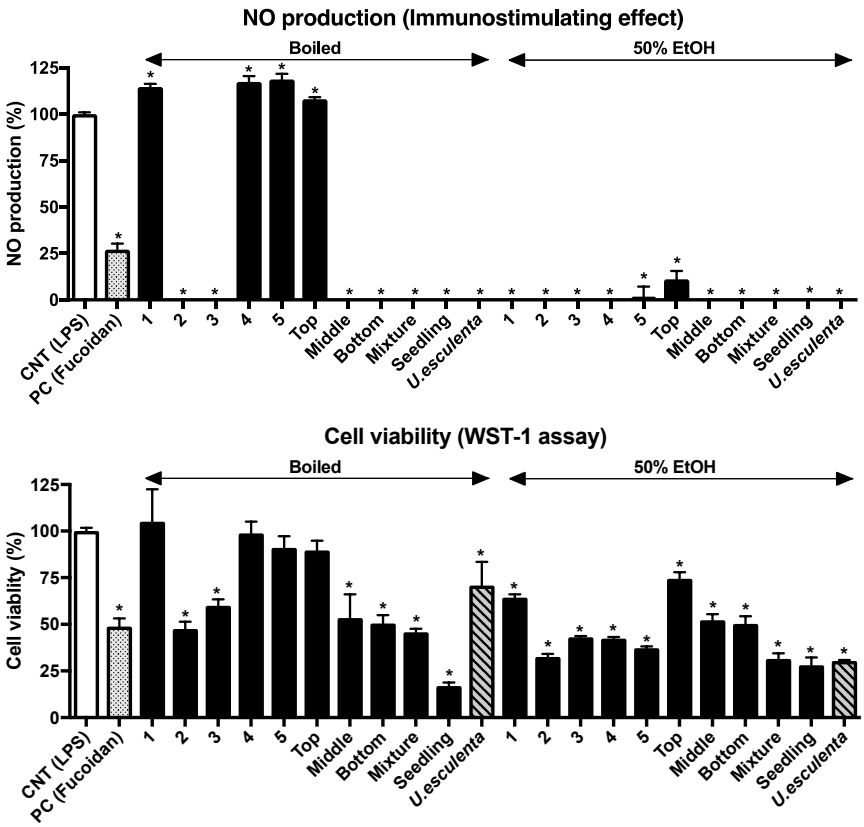

Fig. 2. Immunostimulatory effects (upper) and effects on cell viability (lower) of white bamboo samples. Relative NO production and cell viability are shown for both boiled (left) and 50 % ethanol (right) extracts against the LPS control. Except for the LPS control, all data were obtained in the absence of LPSs. The remaining descriptions are the same as in Fig. 1, except PC, which was a 1 mg mL^{-1} fucoidan solution in this experiment.

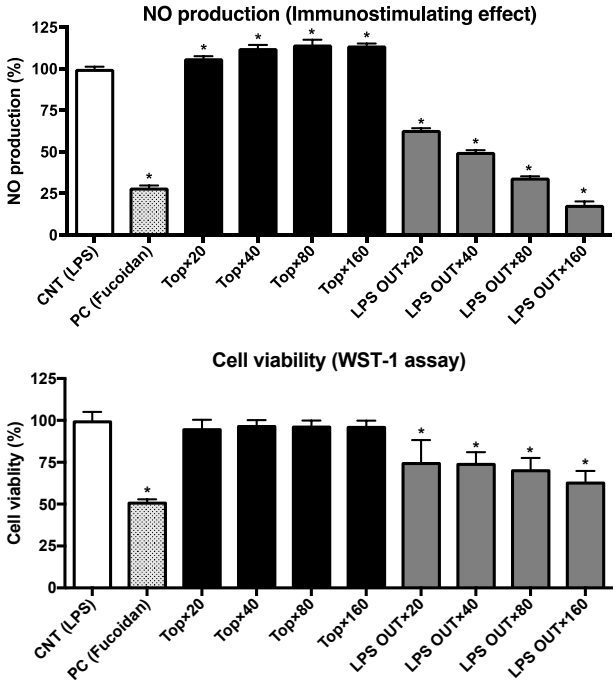

Fig. 3. Immunostimulatory effects (upper) and effects on cell viability (lower) of boiled extracts from the top part (apical side) of white bamboo. "Top x" indicates the dilution level of the original 100 mg g^{-1} extract. "LPS OUT x" indicates the extract solution in which LPSs were removed using an affinity column. The remaining descriptions are the same as in Fig. 2.

with the eluate to some extent (Fig. 3).

TPP concentration The TPP concentrations of the 50 % ethanol extracts varied with swelling stage and white bamboo stem segment (Fig. 4). At the earliest swelling stage, i.e., sample No. 1, the TPP was the highest at 3.24 mg-QE g^{-1} and gradually decreased with stem enlargement. At the time of sporulation (samples No. 4 and No. 5), TPP decreased to nearly half that observed in sample No. 1. Among the different parts of white bamboo, the top part of the stem showed the highest TPP value. Moreover, the TPP of seedlings was the highest of all samples.

DPPH radical scavenging activity The DPPH radical scavenging activity was almost identical throughout the different swelling stages but changed with varying stem segment, with the middle segment yielding the lowest value (Fig. 5). The DPPH activity was highest in the seedlings, as was observed with the TPP.

Discussion

Inflorescence and fruition of *Z. latifolia* are prevented by infection with *U. esculenta.* The seeds of *Z. latifolia* are known as wild rice and are abundant in nutrients and bioactive compounds with health benefits (Yan *et al.,* 2018b). White bamboo is a unique vegetable, not only because of its taste and texture but also because of the fungal-plant interaction (Guo *et al.,* 2007). Functional research on white bamboo first focused on its anti-osteoporotic effect and the responsible N-glycoside compounds makomotindoline (Kawagishi *et al.,* 2006) (Suzuki *et al.,* 2012) and makomotines (Choi *et al.,* 2014). Recent studies have identified immunostimulatory activities of water-

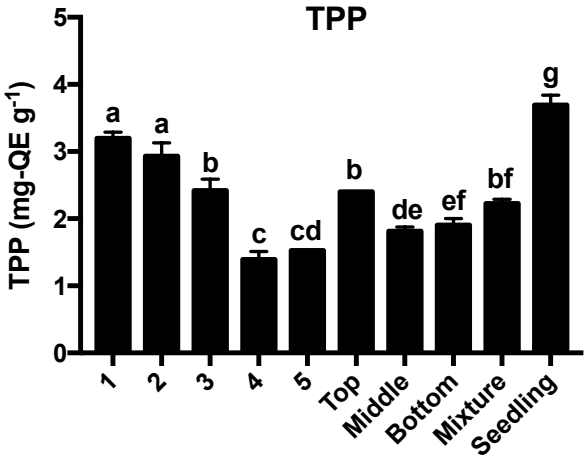

Fig. 4. TPP concentrations in 50 % ethanol extracts of white bamboo samples. Each column represents the TPP concentration in quercetin equivalents (mg-QE g^{-1}) expressed as the mean + standard deviation, n = 2. Different letters indicate significant differences ($p < 0.05$). The samples are identical to those in Fig. 1.

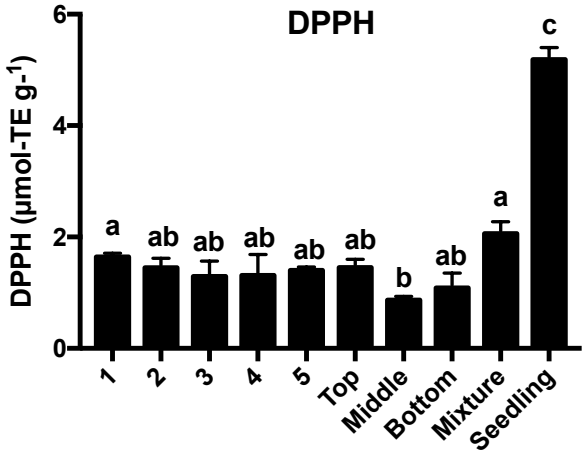

Fig. 5. DPPH radical-scavenging activities of 50 % ethanol extracts of white bamboo samples. Each column represents DPPH activity as Trolox equivalents (µmol-TE g^{-1}) expressed as the mean + standard deviation, n = 2. Different letters indicate significant differences ($p < 0.05$). The samples are identical to those in Fig. 1.

soluble and beta-glycated polysaccharides from white bamboo (Wang *et al.*, 2017) (Wang *et al.*, 2018). These studies focused on the isolation and identification of the responsible chemicals. In this study, we focused on the immunoregulatory function of fungal-plant interactions in terms of the growth stage and white bamboo stem segment.

The anti-inflammatory effect on mouse RAW264 macrophages was comprehensively detected in the 50 % ethanol extracts regardless of swelling stage or stem segment (Fig. 1). These results explain the ubiquitous presence of bioactive anti-inflammatory compounds in white bamboo. The higher activity in the ethanolic extracts than in the boiled extracts, as well as the similar tendency of the TPP concentration and DPPH activity (Figs. 4, 5), support the contribution of glycoside polyphenols, such as makomotines with a typical aglycon, 2-methoxy-4-vinylphenol (Choi *et al.*, 2014), to the anti-inflammatory property of white bamboo.

In contrast, an immunostimulating effect was distinctively detected in the boiled extracts and localized in the earliest sample, No. 1, and in the aged samples, Nos. 4 and 5, throughout swelling; moreover, this immunostimulating effect was only observed in the top part (apical side) of the stems (Fig. 2). Some of the strong substances inducing NO production are LPSs that compose the outer membrane of gram-negative bacteria. In our previous research on the microbiota of white bamboo, gram-negative bacteria, such as *Pantoea* sp., were identified (Tu *et al.*, 2019). The NO-promoting activity was particularly strong even when the extract was diluted 160-fold (Fig. 3). In fact, pure LPSs also persistently exhibited the ability to promote NO production independent of dose (data not shown). When LPSs were removed from the extract, the NO-promoting effect was maintained but reduced and revealed clear dose dependency. This result suggests that LPSs are some of the immunostimulating compounds contained in white bamboo; however, other responsible components are likely present in white bamboo. The other compounds responsible for the maintained immunostimulating activity are likely water-soluble and non-starch polysaccharides with beta-type glycosidic linkages (Wang *et al.*, 2018). Specific accumulation of these polysaccharides and whether it depends on swelling stage of the culm or the segment of the stem of white bamboo should be researched in the future. Measuring the expression of inflammatory cytokines or Toll-like receptors (TLRs) may also contribute to the understanding of the underlying mechanisms or components responsible for these unique effects.

The mixture (whole sample of white bamboo) had no immunostimulating effect, although the top part revealed strong activity (Fig. 2). This may be explained by a balance of antagonistic actions, especially in the boiled extract, between anti-inflammatory and immunostimulating effects since NO production of RAW264 cells was regulated in terms of both inhibition and promotion at the same time (Fig. 6). The top part (apical side) includes the meristem, the most active place in plant tissue where nutrients and metabolites are being

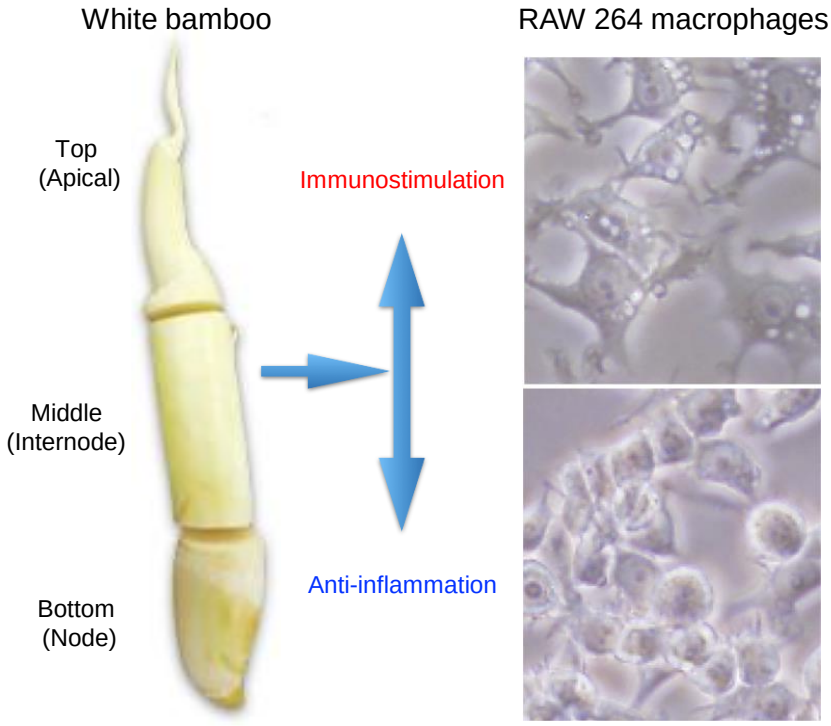

Fig. 6. Simultaneous inhibition and promotion of NO release due to antagonism between anti-inflammatory and immunostimulating effects of white bamboo on RAW264 cells.

exchanged with the parasitized fungus (Chan and Thrower 1980). Immunostimulatory polysaccharides contained in white bamboo might be induced through fungi-plant interactions in the meristem.

Interpretation of the results of a cell-based assay of complex extracts is difficult when considering conflicting aspects of cytotoxicity and cell activation. Cytotoxicity appears when the concentration exceeds a toxic threshold, depending on each component, even though cell activation may also occur within some concentration ranges. Anti-inflammatory activity is also influenced by cytotoxicity or cell activity. The prominent reductions of cell viability by sample No. 2 and the seedlings could be ascribed to their cytotoxic components, to some extent (Fig. 1). No immunostimulating activity was observed for samples Nos. 2 and 3 of boiled extracts, while samples Nos. 1, 4 and 5 clearly increased NO release (Fig. 2). The extracts of samples Nos. 2 and 3 are most likely to reduce cell viability and then diminish their immunostimulating activities. The highest cell viability, shown by the alcoholic extract of the top part among the divided parts, may also explain the reason why NO release was observed only with the top part extract. The removal of LPS from the extract of the top part reduced cell viability by 37 % compared with that in the paired 160-fold diluted sample with LPS (Fig. 3). Thus, LPS is considered to be one of the cell-activating substances.

At the beginning of this study, we assumed that the fungal body of *U. esculenta* may contribute to any immunoregulatory effects. However, no immunostimulating effect was detected from *U. esculenta* itself (Fig. 2), although mild anti-inflammatory activity was observed in the ethanolic extract (Fig. 1). Therefore, we proposed that functionally responsible components, such as makomotines and polysaccharides, are dynamically formed depending on the interaction between *Z. latifolia* and *U. esculenta*, and like the phytohormone indole-3-acetic acid, they may also influence the growth of the plant and symbionts (Chung and Tzeng 2004a).

White bamboo has been consumed as a popular vegetable for centuries in Asian countries. Regarding recent concerns about the health-promoting effect of white bamboo, the top part of the stem could effectively be provided as a dried powder for producing white bamboo tea with an immunostimulatory effect. The residual part could be sliced and soaked in drinking alcohol to produce a white bamboo liquor and would likely exhibit anti-inflammatory effects.

Conclusion

In this study, we evaluated immunoregulatory effects of white bamboo, depending on the swelling stage and the parts of the stem collected at the optimal harvest time, by monitoring NO production by RAW264 macrophages. The results showed that the anti-inflammatory effect was comprehensively detected in the ethanolic extracts. However, immunostimulating activity was observed in the boiled extracts with different intensities for different swelling stages and different white bamboo stem segments, especially in the top part of the stem at optimal harvest time. We proposed that the functional components in white bamboo formed as a result of the interactions between *Z. latifolia* and *U. esculenta*. Further studies, such as focusing on the expression of inflammatory cytokines or TLRs, are necessary to confirm these immunoregulatory effects of white bamboo by overcoming the limitation of the evaluation based on NO alone.

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Conflict of interest There are no conflicts of interest to declare.

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