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Allelopathy of Wild Mushrooms—An Important Factor for Assessing Forest Ecosystems in Japan

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Abstract: Research Highlights: Some organisms such as plants and fungi release certain secondary metabolites, generally called allelochemicals, which can influence the organisms around them. Some of the secondary metabolites released by mushrooms may have certain effects on the growth and development of neighboring plants. Background Objectives: The purpose of the present study was to investigate the allelopathic potential of mushrooms in a forest ecosystem. To this end, 289 Japanese mushroom species were collected from the wild and tested using a modified sandwich method, which is a quick and effective bioassay technique. Materials and Methods: The collected specimens were prepared for bioassay as dried samples, and 10 mg/well (10 cm^2) was added to a 6-well multidish according to the mycelia biomass, which was estimated at 700−900 kg ha^{-1} $year^{-1}$ (7–9 mg 10 cm^{-2}) in coniferous forests. Results: Of the screened mushroom species, 74% inhibited more than 50% of the radicle elongation in lettuce (*Lactuca sativa* var. Great Lakes 366) seedlings, while the average of all species was 41.1%. This result suggests that wild mushrooms have a significant regulatory effect on lettuce growth. According to our standard deviation variance analysis, 54 out of 289 species showed significant allelopathic activity. Among these species, *Xeromphalina tenuipes*, *Cortinarius violaceus*, and *Clavaria miyabeana* exhibited the strongest growth inhibitory activity, with radicle elongation of 5.1%, 4.3%, and 7.6% of the control, respectively. In contrast, *Ischnoderma resinosum* stimulated the length of radicle and hypocotyl growth by 30.6% and 42.0%, respectively. These results suggest that these species may play important roles in ecosystems. In addition, the wide range of allelopathic activities observed in mushrooms indicates that various amounts of diverse secondary metabolites from these species are involved in mushroom allelopathy. Conclusions: Our study reveals the importance of evaluating mushroom allelopathy to understand the wider ecological structures within complex ecosystems.

Keywords: mushroom allelopathy; sandwich method bioassay; plant growth regulation; forest ecosystem

1. Introduction

A forest ecosystem is a complex of living organisms. All of them interact with the environment in which they live and among themselves via various bioactive chemicals [1]. Allelopathy through the release of allelochemicals is one of the ecological interactions which effects the growth

and development of forest ecosystems. Allelochemicals are directly and indirectly involved in seedling growth disturbances, including the delay and reduction of germination and restriction of root development. In mature trees, mycorrhizal functions and growth stagnation may result in death and the disappearance of species in severe cases. Blanco [2] stated that plant allelopathy is an important ecological factor in forest ecosystems. Allelopathic plants may promote succession in non-native environments by possessing unique bioactive compounds [3]. Accordingly, allelopathy may be much more important as a mechanism in recipient natural plant communities because they appear to evolve tolerance to chemicals [4]. However, neither Blanco nor others have considered the allelopathy of mushrooms, including mycorrhizal fungi, as important factors. This is in spite of the substantial evidence on the interference of allelopathic mushrooms in natural ecosystems through unique allelochemicals [5,6].

Higher fungi—Ascomycetes and Basidiomycetes—interfere in many ecosystems with nearby plants, either directly or indirectly [7–10]. Basidiomycete mushroom aqueous extracts can directly inhibit/stimulate the growth and germination rates of *Pinus banksiana* as well as lichens, herbaceous plants, and trees [11]. Furthermore, mycorrhizal mushrooms have a symbiotic relationship with certain trees to exchange bioactive chemicals. These chemicals, when released into the environment, may show certain allelopathic effects on neighboring non-symbiotic plants [12]. Additionally, saprotrophic mushrooms produce many phenolic allelochemicals by decomposing lignin in wood litters [13]. Thus, fungal material should also be considered to gain a clearer understanding of ecosystems. Plant litter biomass accounts for approximately 1500–2500 kg ha^{-1} $year^{-1}$ in coniferous forests [14], while the amount of ectomycorrhizal mycelia biomass has been estimated at 700–900 kg ha^{-1} $year^{-1}$ [15]. This represents a significant source of allelochemicals which are released into the soil organic layer. In a recent review on mushroom allelopathy, Araya [16,17] proposed that the routes of release for allelochemicals from mushrooms were similar to those in plants. The allelopathic activity of mushrooms depends on hyphae biomass and on the amount/strength/variety of allelochemicals, in addition to the tolerance of affected organisms (e.g., plants or insects) [18]. Accordingly, using wild filamentous hyphae with fruiting bodies would be ideal for assessing the allelopathic potential of mushrooms. Because it is impossible to collect large amounts of wild filamentous hyphae for bioassay, the only available option is to use fruiting bodies, collectable aggregates of wild hyphae [16,17].

A simple and quick bioassay procedure is necessary to evaluate the allelopathic potential of a large number of mushroom samples. Several researchers have developed such methods to evaluate the allelopathic properties for individual plants/compounds [18–26]. These methods were designed based on the three main allelochemical release routes of plants: leaf litter leachates, roots exudates (in vitro and roots exudates in the soil), and volatiles. In the present study, we used the sandwich method to evaluate the allelopathic potential of the fruiting bodies of 289 macro-fungi from Japan [16,17,27]. Lettuce (*Lactuca sativa* var. Great Lakes 366) was used as a test plant to represent the seedling growth disturbance, due to the advantage of its susceptibility and uniform growth response to allelochemicals. Consequently, information on the inhibitory or stimulatory effects of chemicals eluted from mushroom fruiting bodies lead to further considerations about mushroom allelopathy in the forests of Japan. Thus, a presumption was made that mushrooms produced allelopathic compounds that affect a test plant directly. The intention of this research is to assess the allelopathic potential of mushrooms and to discuss the necessity of mushroom allelopathy as one of the important factors in forest ecosystems.

2. Materials and Methods

2.1. Fungal Collection

More than 800 kinds of mushroom fruiting bodies were collected from various geographical locations in 24 prefectures in Japan between May 2000 and August 2016 (Figure 1). Taxonomic identification was carried out by two of the authors (Ishizaki and Araya) through

morphological examination (specimen numbers provided at supplementary data). Mushroom samples of 670 fruiting bodies among them were categorized into the 289 species that were used in the study. All the collected fruiting bodies were cleaned and lyophilized, then finely pulverized and bottled for storage in a dark freezer (Sanyo, BioMedical Freezer MDF-U536D, Osaka, Japan). Each year, a bioassay was conducted on the mushrooms collected in that year. All collected mushroom species were stored in the Natural Products Chemistry Laboratory, School of Agriculture, Meiji University.

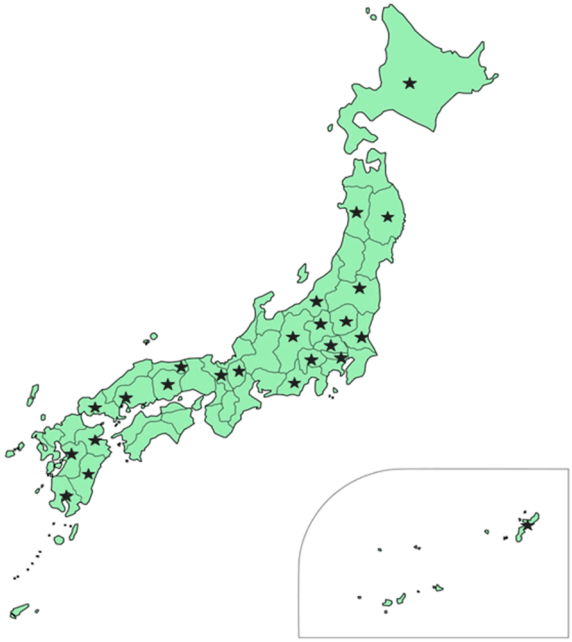

Figure 1. Locations of sampling sites in Japan: 24 prefectures indicated by stars.

2.2. Bioassay of Allelopathic Activity of Mushroom Fruiting Bodies Using the Modified Sandwich Method

Samples were screened using the modified sandwich method, which was developed in our previous studies [14,19]. In the sandwich method, 10 or 50 mg of plant material is placed in each well of a 6-well plastic multidish. For mushrooms, however, our previous study showed that 10 mg of dried material was more suitable due to the strength of the activity observed with 50 mg [16,17]. Despite the fact that 50 mg is the minimum amount for plant material, 10 mg of mushroom powder is almost the maximum amount, based on our knowledge of natural conditions as discussed above. Low-temperature gelling agar (0.75% w/v) was used as a growth medium (Nacalai Tesque, Kyoto, Japan; gelling temperature from 30 to 31 °C). The agar was autoclaved at 115 °C for 15 min and then cooled to 40 °C in a water bath. The mushroom powder (10 mg) was placed in each well, and then 5 ml of the agar was added using an auto-pipette (Gilson Co. Ltd, Villiers-le-Bel, France). If the samples floated on the agar, then they were submerged in the agar using micro-spatulas until the agar gelled. After the complete gelatinization of the agar layer (within 30–60 min at room temperature), another 5 mL of agar was added over the first layer in each well and left for 60 min to solidify. In this way, the lower agar layer contained the mushroom materials and was shielded by the second, pure agar layer. Lettuce (*Lactuca sativa* L. Great Lakes 366, Takii Seed Co. Ltd, Kyoto, Japan) was used as the test plant due to its germination reliability, rapid and uniform growth response, susceptibility to chemicals, and exogenous bioactive compounds [28]. Five lettuce seeds were placed on the surface of each agar-containing well of the plate. This allowed for adequate water content for growth and covered the whole area of each well with plant material with adequate distance for their growth. The multidishes were sealed with plastic tape to prevent dehydration and incubated (BIOTEC 300-L, Shimadzu Rika Institute Co. Ltd, Kyoto, Japan) for 72 h at 25 °C in the dark.

The lengths of the radicles and hypocotyls were measured with 1 mm accuracy. Each experiment was replicated three times and the results presented are the mean of the results of these three replicates.

2.3. Statistics

The percentage of the radicle and hypocotyl growth ratio of the lettuce seedlings was calculated for each sample compared with the control by the following formula:

$$\text{Growth ratio}(\%) = 100 \times (\text{average of sample length}/\text{average of control length})$$

To measure the growth ratio of each of the 289 mushroom species, averages were calculated within the same species samples in advance.

For the evaluation of allelopathic activity among individual samples, the concept of the "standard deviation variance" was applied [12,29] to show how much individuals can vary from total mean of our normal population. The mean (m) and standard deviation (σ) were calculated and the criterion of the standard deviation variance (SDV) was estimated to rank the species that exhibited significant effects. Criteria indices (*: m−σ, **: m−1.5σ, ***: m−2σ, and ****: m−2.5σ) indicate that the radicle and hypocotyl growth rate data can be combined to provide a unique index for ranking all mushrooms. The number of samples that regulated lettuce seedling growth was counted within the range of 10% regulation. In addition, correlation between growth average ratios of radicle and hypocotyl was analyzed for all 16 fungal orders collected, in parallel with their total average of growth regulation.

3. Results

A total of 670 mushroom fruiting bodies belonging to 289 species of 16 orders of macro-fungi were identified and screened for allelopathic effects on the growth of lettuce seedlings. The results of both lettuce radicle and hypocotyl growth conformed to normal distribution (radicle kurtosis = 1.69 and skewness = 1.05; hypocotyl kurtosis = 0.71 and skewness = 0.27), and it was observed that radicle elongation was affected by allelopathic compounds more than the hypocotyl (Figure 2). The radicle and hypocotyl growth percentages of lettuce seedlings were in the range of 2.8–131% and 11.4–147% of the control, respectively. A range of lettuce radicle elongation from 0 to 30% was observed in 30 species, from 30 to 50% in 125 species, from 50 to 70% in 90 species, from 70 to 90% in 27 species, and from 91–100% in eight species. The remaining eight species exhibited stimulation effects (101–150%), according to standard deviation variance (SDV) with 99% confidence (Figure 2a). In the case of lettuce hypocotyl elongation, a range of 0–30% was observed in just one species, 30–50% in 13 species, 50–70% in 42 species, 70–90% in 109 species, and 91–100% in 48 species. The remaining 75 species stimulated lettuce hypocotyl growth (101–150%) (Figure 2b).

As shown in Figure 2a, 163 mushrooms inhibited lettuce radicle elongation up to the average point of 41.1%, and 147 mushrooms inhibited lettuce hypocotyl growth to the average point of 76.8% (Figure 2b). Of the screened mushroom species, 74% inhibited lettuce radicle growth over 50% (Figure 2a). In addition, only 12% of the mushroom species in this study inhibited hypocotyl growth by 50% (Figure 2b). A previous study, which examined 81 species (including cultivated specimens), reported that approximately 80% of the mushrooms inhibited radicle growth by 50% at the same application rate, and almost all of the species examined inhibited lettuce hypocotyl growth at various ranges [30].

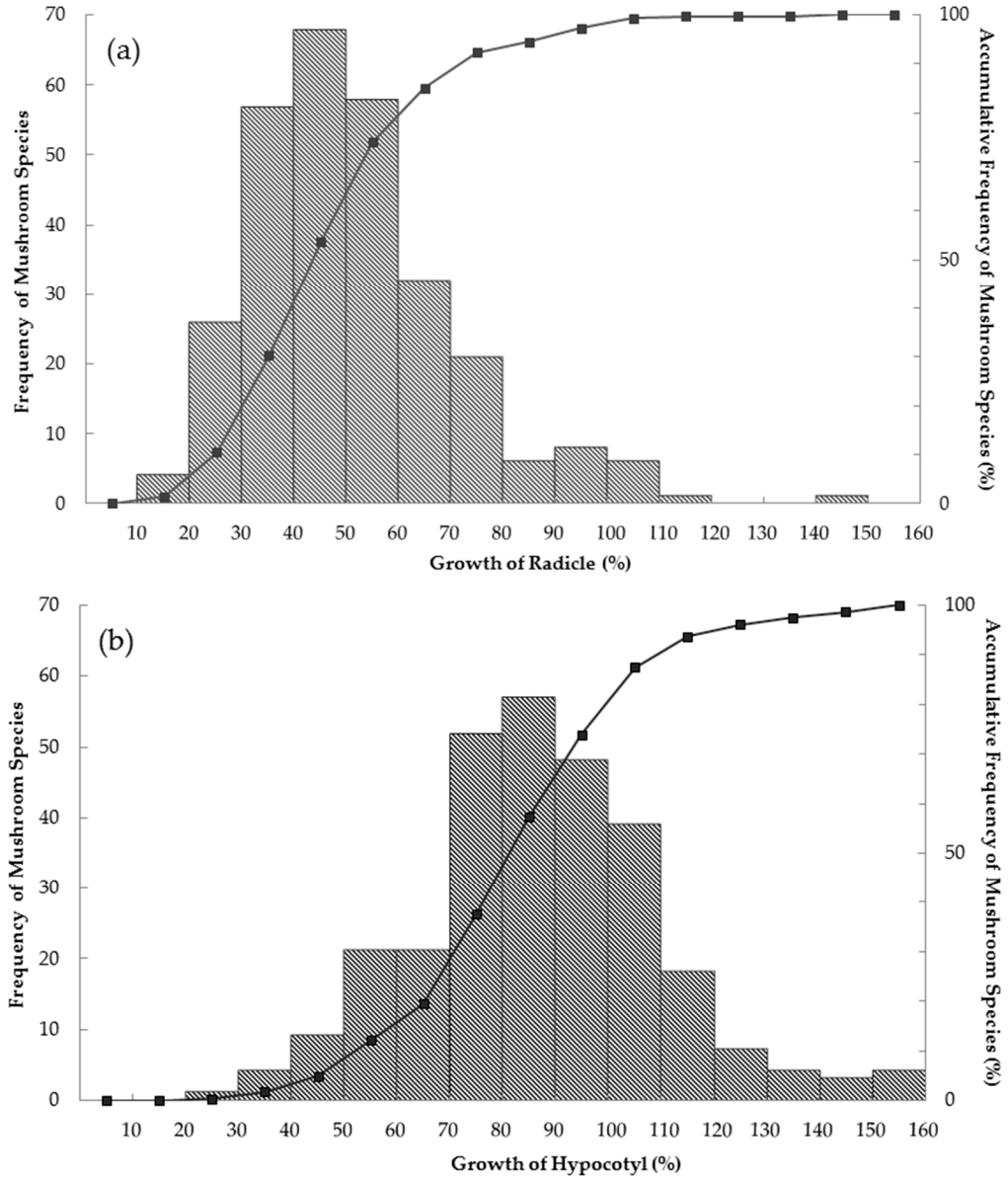

Figure 2. Probability density of the allelopathic effect of the 289 mushroom species on lettuce (*Lactuca sativa* var. Great Lakes 366) radicle growth (**a**) and hypocotyl growth (**b**), with the accumulative distribution of the number of involved species also illustrated.

The correlation between radicle and hypocotyl growth, as illustrated in Figure 3, suggests that radicle growth was inhibited to a higher degree than hypocotyl growth. Moreover, the high coefficient of the correlation (r = 0.809) suggests that the inhibition of the growth of the radicle was more affected than the hypocotyl, which indicates that the radicle is more susceptible to this.

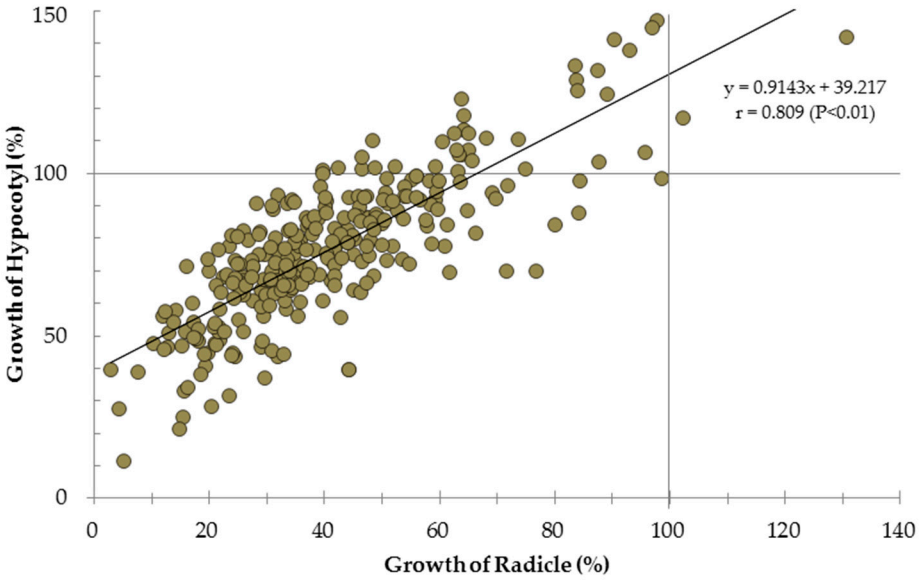

Figure 3. Correlation between radicle and hypocotyl growth of lettuce.

The standard deviation variance analysis suggested that the 54 species listed in Table 1 significantly inhibited both radicle and hypocotyl growth. With reference to radicle growth, *Calocybe gambosa* (2.8%), *Cortinarius violaceus* (4.3%), *Xeromphalina tenuipes* (5.1%), *Clavaria miyabeana* (7.6%), and *Heimiella japonica* (10.2%) showed the highest levels of inhibition of radicle elongation. Moreover, we observed that *X. tenuipes* (11.4%), *Leucopaxillus septentrionalis* (21.4%), *Pholiota spumosa* (25.1%), *C. violaceus* (27.4%), and *Entoloma clypeatum* (28.4%) exhibited the highest levels of hypocotyl elongation inhibition (Table 1). Conversely, the results based on the total estimation of criterion (99% confidence) showed that *X. Tenuipes*, radicle (*R*) = 5.1% and hypocotyl (*H*) = 11.4%, followed by *C. violaceus*, *R* = 4.3% and *H* = 27.4%, *C. miyabeana*, *R* = 7.6% and *H* = 38.8%, *C. gambosa*, *R* = 2.8% and *H* = 39.8%, *E. clypeatum*, *R* = 20.3% and *H* = 28.4%, *P. Spumosa*, *R* = 15.4% and *H* = 25.1%, and *L. septentrionails*, *R* = 14.8% and *H* = 21.4%, inhibited both radicle and hypocotyl elongation, similar to a previous study [31]. The strongest growth-inhibiting species belonged to Mycenaceae, Cortinariaceae, Clavariaceae, Lyophyllaceae, Entolomataceae, Strophariaceae, and Tricholomataceae of the phylum Basidiomycota (Table 1). Moreover, isolated bioactive compounds and the reported bioactivities of listed mushrooms are shown in Table 1.

Several species of tested mushrooms caused a slight stimulatory activity on the radicle and hypocotyl growth, which are plotted out of 100% as shown in Figure 3. Among these species, *Ischnoderma resinosum* and *Exidia glandulosa* stimulated the radicle and hypocotyl growth of lettuce to *R* = 130.6% and *H* = 142%, and *R* = 102.2% and *H* = 117%, respectively (Figure 3). This strong stimulatory activity of *I. resinosum* and *E. glandulosa* suggests that some allelochemical(s) can stimulate the growth of lettuce seedlings.

The SDV and mean with 99% confidence were calculated to assign various criteria (radicle growth and hypocotyl growth) to indicate significant inhibition levels among the mushroom species. Among the 289 species, the 54 species listed in Table 1 showed significant inhibition to merit criteria indices that indicate the level of growth inhibition.

Table 1. Characteristics of 54 significant Japanese mushroom species ranked by criterion index.

Orders	Family	Species	Prop.	Pthg.	R. Grw.	H. Grw.	Criteria	Bio. Act. & Bi. Com.
Agaricales	Mycenaceae	*Xeromphalina tenuipes*	Sapro.	No	5.1	11.4	******	NR
Agaricales	Cortinariaceae	*Cortinarius violaceus*	Myco.	No	4.3	27.4	*****	Cysteine protease inhibitor, (*R*)-*β*-dopa, *β*-glucans [32,33]
Agaricales	Clavariaceae	*Clavaria miyabeana*	Myco./Sapro.	NR	7.6	38.8	****	L-azetidine-2-carboxylic acid [34]
Agaricales	Lyophyllaceae	*Calocybe gambosa*	Sapro.	Yes	2.8	39.8	****	Fairy ring [35]
Agaricales	Entolomataceae	*Entoloma clypeatum*	Myco.	No	20.3	28.4	****	NR
Agaricales	Strophariaceae	*Pholiota spumosa*	Sapro.	No	15.4	25.1	****	(*R*)-2-hydroxyputrescine dicinnamamide [36]
Agaricales	Tricholomataceae	*Leucopaxillus septentrionalis*	Sapro.	No	14.8	21.4	****	Antibiotic activity (clitocine), antioxidant activity [37]
Boletales	Boletaceae	*Heimiella japonica*	Myco.	No	10.2	47.7	***	NR
Agaricales	Coprinaceae	*Coprinus comatus*	Sapro.	No	15.6	33.2	***	Fucogalactan, antimicrobial activity, nematophagous [38]
Agaricales	Entolomataceae	*Entoloma abortivum*	Para./Sapro.	No	19.4	40.7	***	NR
Agaricales	Entolomataceae	*Entoloma sarcopum*	Myco.	No	18.5	38.0	***	NR
Agaricales	Marasmiaceae	*Pleurocybella porrigens*	Sapro.	No	16.2	34.0	***	Aziridine, cyanide salt [39,40]
Agaricales	Tricholomataceae	*Clitocybe clavipes*	Sapro.	No	23.5	31.5	***	Acetaldehyde dehydrogenase inhibitor [41]
Agaricales	Agaricaceae	*Agaricus arvensis*	Sapro.	No	13.0	50.9	**	Fairy ring, sinapic acid [42]
Agaricales	Amanitaceae	*Amanita pseudoporphyria*	Myco.	No	15.8	51.3	**	Nephrotoxin [43]
Agaricales	Amanitaceae	*Amanita sinensis*	Myco.	No	17.3	54.2	**	NR
Thelephorales	Bankeraceae	*Sarcodon scabrosus*	Myco.	No	12.2	45.6	**	Neosarcodonin, 4-Methoxy-6-phenyl-2*H*-pyran-2-one [44,45]
Boletales	Boletaceae	*Boletellus floriformis*	Myco.	No	21.1	47.4	**	NR
Boletales	Suillaceae	*Boletinus paluster*	Myco.	No	21.0	53.9	**	Xerocomic acid, variegatic acid, variegatorubin [46]
Boletales	Boletaceae	*Boletus quercinus*	Myco.	No	13.8	54.4	**	NR
Boletales	Boletaceae	*Xanthoconium affine*	Myco.	No	20.7	52.7	**	NR
Russulales	Bondarzewiaceae	*Bondarzewia mesenterica*	Sapro.	Yes	17.3	49.3	**	Montadial A [47]
Agaricales	Hydnangiaceae	*Laccaria vinaceoavellanea*	Myco.	No	12.8	46.4	**	Hemolytic toxins, laccarin (I) [48,49]
Agaricales	Hygrophoraceae	*Hygrophorus erubescens var. capreolarius*	Myco.	No	19.7	44.8	**	Harmane and norharmane [50]
Agaricales	Hygrophoraceae	*Hygrophorus pudorinus*	Myco.	No	19.2	44.5	**	Antibacterial activity [51]
Agaricales	Inocybaceae	*Inocybe lutea*	Myco.	No	18.1	48.3	**	NR
Polyporales	Polyporaceae	*Polyporus squamosus*	Para./Sapro.	No	17.7	49.0	**	Antioxidant and antimicrobial activity [52]
Agaricales	Tricholomataceae	*Clitocybe candicans*	Sapro.	No	21.0	47.5	**	Candicansol, *epi*-illudol and 1-*O*-acetyl-3-*epi*-illudol [53]
Agaricales	Tricholomataceae	*Clitocybe nuda*	Sapro.	NR	18.0	51.9	**	Antibacterial (2-methoxy-5- methyl-6-methoxy-methyl-*p*-benzoquinone, 6-hydroxy-2*H*-pyran-3-carbaldehyde and indole-3-carbaldehyde) [54]
Agaricales	Tricholomataceae	*Tricholosporum porphyrophyllum*	Sapro.	NR	15.2	47.0	**	NR
Gomphales	Gomphaceae	*Gomphus purpuraceus*	Myco.	No	29.6	37.1	**	Purpuracolide [55]
Gomphales	Gomphaceae	*Ramaria fennica*	Myco.	No	44.3	39.5	**	NR

Table 1. *Cont.*

Orders	Family	Species	Prop.	Pthg.	R. Grw.	H. Grw.	Criteria	Bio. Act. & Bi. Com.
Agaricales	Physalacriaceae	*Armillaria tabescens*	Sapro.	Yes	44.3	39.5	**	Bioluminescence,4-dehydro-14-hydroxy-dihydromelleolide, 4-dehydro-dihydro-melleolide, 14-hydroxy-dihydromelleolide, 13-hydroxy-4-methoxy-melleolide and 5*β*,10α-dihydroxy-l-orsellinate-dihydromelleolide, emestrin-F, emestrin-G, 6-*O*-(4-*O*-methyl-*β*-*d*-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4*H*-benzopyran-4-one, purpuracolide, and cephalosporolide-J [56–58]
Agaricales	Agaricaceae	*Lanopila nipponica*	NR	NR	20.0	69.8	*	NR
Agaricales	Agaricaceae	*Lycoperdon pratense*	Sapro.	No	16.1	71.5	*	NR
Auriculariales	Auriculariaceae	*Exidia uvapassa*	Sapro.	No	21.1	65.7	*	NR
Agaricales	Cortinariaceae	*Cortinarius caperatus*	Myco.	No	19.7	73.7	*	NR
Agaricales	Pluteaceae	*Pluteus leoninus*	Sapro.	No	14.1	57.9	*	NR
Polyporales	Polyporaceae	*Microporus vernicipes*	Sapro.	No	12.3	57.6	*	NR
Russulales	Russulaceae	*Lactarius subvellereus*	Myco.	No	17.0	60.1	*	Subvellerolactones [59]
Agaricales	Tricholomataceae	*Lepista graveolens*	Sapro.	No	11.9	56.0	*	NR
Agaricales	Agaricaceae	*Agaricus subrutilescens*	Sapro.	No	21.8	48.6	*	L-α-amino-γ-nitraminobutyric acid [60]
Auriculariales	Auriculariaceae	*Auricularia minor*	Sapro.	No	29.1	48.3	*	Antitumor activity [61]
Boletales	Boletaceae	*Leccinum versipelle*	Myco.	No	21.6	51.3	*	NR
Polyporales	Fomitopsidaceae	*Phaeolus schweinitzii*	Sapro.	Yes	33.0	44.3	*	Antitumor and radical-scavenging activity, Hispidin, pinillidine [62,63]
Agaricales	Hygrophoraceae	*Hygrophorus speciosus*	Myco.	No	31.8	43.6	*	NR
Agaricales	Hygrophoraceae	*Hygrophorus hypothejus*	Myco.	No	24.4	43.7	*	Anti-(A+B) blood type specific lectin, 1,2-diacylglycero-*O*-4′-(*N*,*N*,*N*-trimethyl) homoserine [64,65]
Phallales	Phallaceae	*Kobayasia nipponica*	Myco.	No	22.7	51.2	*	*β*-Glucuronidase [66]
Agaricales	Physalacriaceae	*Armillaria gallica*	Sapro.	No	21.6	52.9	*	Bioluminescence [67]
Russulales	Russulaceae	*Russula neoemetica*	Myco.	No	23.9	44.1	*	NR
Agaricales	Strophariaceae	*Hypholoma fasciculare*	Sapro.	No	28.9	46.7	*	Bactericidal effects [68]
Agaricales	Strophariaceae	*Pholiota terrestris*	Sapro.	No	26.0	51.2	*	Highest cellulase and laccase activities among Pholiota genius [69]
Agaricales	Tricholomataceae	*Melanoleuca verrucipes*	Sapro.	No	24.0	44.6	*	Glucans [70]
Agaricales	Tricholomataceae	*Tricholoma auratum*	Myco.	No	30.9	45.4	*	(22*E*,24*R*)-Ergosta-7,22-diene-3*β*,5α,6β-triol [71]
Mean (m)					41.1		76.8	
Standard deviation (σ)					19.7		22.3	
m−σ (*)					21.4		54.5	
m−1.5σ (**)					11.5		43.4	
m−2σ (***)					1.69		32.2	
m−2.5σ (****)							21.1	

Bi. Com.: Bioactive compounds; Bio. Act.: Bioactivity; H. Grw.: Hypocotyl growth; Myco.: Mycorrhizal; Para.: Parasitic; Prop.: Properties; Pthg.: Phytopathogenecity; R. Grw.: Radicle growth; Sapro.: Saprotroph; NR: No records. Criteria indices (*: m−σ; **: m−1.5σ; ***: m−2σ; ****: m−2.5σ) indicate that the radicle and hypocotyl growth rate data can be combined to provide a unique index (i.e., *X. tenuipes R*= ** (m−1.5σ) and *H*= **** (m−2.5σ) combined to ******).

The correlation between the hypocotyl and radicle growth regulation of lettuce by fungal orders is illustrated in Figure 4. Despite the varying number of collected mushrooms for each order, the order Corticiales exhibited the highest average growth inhibition activity (R = 24%; H = 66.2%) while Hymenochaetales presented the lowest growth inhibition activity (R = 65.7%; H = 99.3%). The order Agaricales had the highest number of species in this study (135), and four orders had only one species. The growth regulatory activity of mushroom orders on lettuce radicle and hypocotyl indicate that they follow a linear trend, suggesting that hypocotyl growth is less affected than radicle growth (Figure 4).

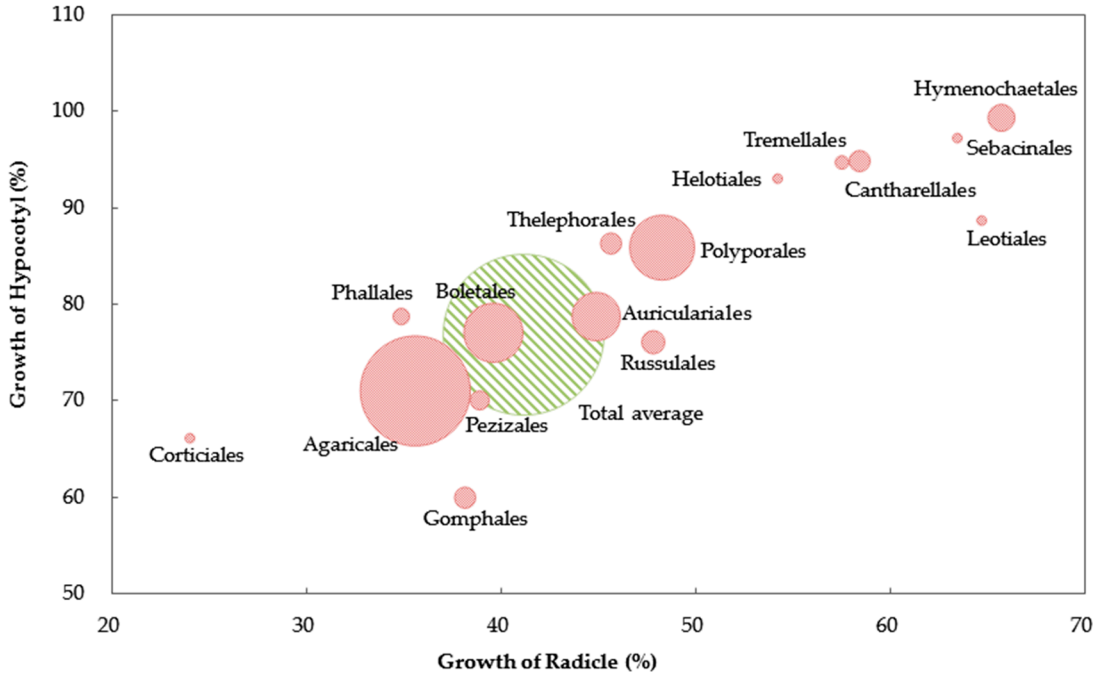

Figure 4. Correlation of radical and hypocotyl growth regulation with fungal orders. The radius of each circle is an indicator of the frequency of screened species in that specific order. The total average is shown with a cross-hatched circle.

The 16 mushroom orders collected included 52 families. Boletaceae had the highest number of species (64 species), followed by Tricholomataceae (54 species), Strophariaceae (53 species), Polyporaceae (51 species), Amanitaceae (43 species), Agaricaceae (42 species), and Russulaceae (38 species) (supplementary data). There was no correlation, however, between mushroom families and their corresponding average of allelopathic activity.

4. Discussions

The sandwich method imitates allelopathy in natural conditions, i.e., the release of allelochemicals from litters; more so than using solvent extraction, because many of the less polar compounds present in extracts prepared using organic solvents are not released under natural conditions. Small pieces of herbaceous plant or tree leaves are used for the sandwich method, to mimic the fallen leaves and water-soluble contents that are extracted by rain without decomposition. Mushroom powder is easier to extract owing to the shorter existence time of fruiting bodies, the smaller size of mycelia than plant tissues, and the lack of hard tissue to decompose. Therefore, we consider that the result of the modified sandwich method using the powder of mushroom fruiting bodies reflects the true mushroom allelopathic potential. Normally distributed modified sandwich method data of 289 mushroom species showed that radicle growth was 41.1% and hypocotyl growth was 76.8% of their normal growth (Figure 2). This result indicates that mushrooms are remarkably stronger growth inhibitors than higher plants (R = 67.3%; H = 109%; growth average of 660 plants in literatures) [29,31,72]. In fact,

plants generally have a larger aerial mass than mushrooms, and higher amounts of allelochemicals are produced in their reproductive organs than in mushroom fruiting bodies. However, the difference between the underground mass of plants and mushrooms is hard to measure, due to their different morphological states.

It is well known that some phytopathogenic fungi and fairy ring mushrooms produce specific phytotoxic secondary metabolites, such as brassicicolin, cyperin, imidazole-4-carboxyamide, and armillaridin; also, these metabolites can act as allelochemicals [73–77]. Although 119 species among the 289 screened species have been recorded as phytopathogenic (see the supplementary data) [75,76], only four species (*C. gambosa*, *Bondarzewia mesenterica*, *Armillaria tabescens*, and *Phaeolus schweinitzii*) among the 54 highly inhibitory species (Table 1) are phytopathogenic fungi. *C. gambosa* is a fairy ring fungus; it regulates the growth of neighboring plants and can be found under trees such as oak, beech, and conifer [35]. *B. mesenterica* is a strong saprotrophic decomposer and is phytopathogenic in wild forests in terms of its specific secondary metabolite, montadial A. Montadial A exhibits therapeutic cytotoxic activities against HL-60 cells [47] and is also a potential allelochemical. Many common trees and shrubs become infected by *A. tabescens*, which causes lethal root rot in coniferous forests as the most common phytopathogenic fungus [56,77,78]. Melleolides have been isolated from *A. tabescens* [56,57] and its analogues have been reported to have inhibitory effects on the growth of lettuce seedlings [5]. Melleolides may also act as allelochemicals. *P. schweinitzii* causes a major disease for old trees by limiting root growth [79] and may produce some root-specific phytotoxic compounds, although there are no reports of the allelopathic activity of hispidin and pinillidine, other isolates from *P. schweinitzii* [62,63], despite their potential plant growth-inhibiting effects. Approximately 40% of the 289 screened mushroom species were phytopathogenic, and these species may produce phytotoxins which may be allelopathic in certain ecosystems. This could be evidence to suggest that some phytotoxins from phytopathogenic mushrooms induce strong effects on their neighbor plants. To confirm this, further experiments testing a variety of plants should be carried out.

The results have shown that most of the wild mushrooms inhibited lettuce radicle and hypocotyl elongation, but in some cases, certain species stimulated lettuce growth. Radicle and hypocotyl growth percentages were in the range of 2.8–131% and 11.4–147%, respectively. In conclusion, radicle growth inhibition was stronger than hypocotyl growth inhibition. This was within expectations, since the radicle is more susceptible to allelochemicals due to its early emergence and direct exposure to the chemicals [80,81]. Moreover, the significant correlation between radicle and hypocotyl growth indicates that the difference between the inhibition of hypocotyl and radicle growth was induced by the mushrooms. Consequently, the modified sandwich method for the initial screening of allelopathic potential in mushrooms was found to be statistically reliable. The result will be applicable to discuss the influence of mushroom allelopathy in forest ecosystems. Further survey for evaluating the specific responses of forest plants to mushroom allelochemicals is necessary, despite the subsequent limitations of them (seed dormancy and uneven/varied germination). Another notable finding was that the most frequent mushroom orders did not show strong allelopathic activity compared with the rare orders. To clarify this finding, further experimental study should be carried out.

5. Conclusions

The modified sandwich method is applied as an accurate and inexpensive methodology for the initial screening of many kinds of mushroom samples, and our results conformed to normal distribution. Bioassay would also help overcome the current issue of applying naturally grown hyphae of wild mushrooms to studies of allelopathy. The screening results showed the presence of high levels of allelopathic effects on lettuce growth, suggesting that these mushrooms may exhibit such regulatory effects in their natural ecosystems. *X. tenuipes* and *C. violaceus* (both edible) were the top inhibiting species, and their potential allelochemicals could be exploited for sustainable weed management.

The average inhibition of lettuce seedling elongation by mushrooms in this study was higher than that reported for plants in other studies. Therefore, the results suggest that mushrooms play an important role in the regulation of forest ecosystems through allelopathy. In this manner, this report provides fundamental information on the allelopathic potentials of mushroom species in the wild for the analysis of natural ecosystems. Overall, our study reveals that allelopathy researchers should consider mushroom allelopathy as an important key to understanding ecological structures. Future research should focus on the isolation and identification of the specific allelochemicals involved in the most promising species of mushroom, as well as the mechanisms of their effects on target plant species.

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