Research Article

Influence of strains and environmental cultivation conditions on the bioconversion of ergosterol and vitamin D_2 in the sun mushroom

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Abstract

BACKGROUND: The fungus *Agaricus subrufescens* is grown commercially in China, the USA, Brazil, Taiwan and Japan, among others. However, each country adopts a cultivation system that significantly influences the agronomical parameters and chemical composition of the harvested mushrooms. In this study, the influence of the cultivation process on the content of ergosterol and vitamin D_2 was evaluated.

RESULTS: Four commercial strains of *A. subrufescens* (ABL 04/49, ABL CS7, ABL 18/01 and ABL 19/01) and two environmental cultivation conditions (in the field and a controlled chamber with the absence of sunlight) were used. In field cultivation, ABL CS7 and ABL 19/01 strains presented better agronomic parameters, whereas in a protected environment ABL 19/01, ABL 04/49 and ABL 18/01 demonstrated better performance, respectively. The highest biological efficiency value (64%) was provided by ABL 19/01 strain in a controlled environment.

CONCLUSION: The highest content in ergosterol (990 mg kg^{-1}) and vitamin D_2 (36.8 mg kg^{-1}) were observed in mushrooms obtained in the field from strain ABL 04/49, which presents reasonable agronomic parameters for cultivation.
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Keywords: *Agaricus subrufescens*; production; field; controlled chamber; quality screening of mushroom

INTRODUCTION

World mushroom production has been growing significantly, from 5 million tons (MT) in 1994 to 35 MT in 2013, representing a sevenfold increase.[1] Five countries lead the production of mushrooms: China (34.8 MT), USA (0.421 MT), Netherlands (0.300 MT), Poland (0.280 MT) and Spain (0.166 MT).[2] Such an increase in mushroom productivity can be assigned to the development of sustainable and economic profitable technologies, which rely on the cultivation in a smaller physical space, with shorter harvest cycles, maintaining sustainability and economic viability during all seasons of the year.[3] *Agaricus subrufescens* Peck is grown commercially in China, the USA, Brazil, Taiwan and Japan, among others,[4] but each country adopts a different cultivation system, which significantly influences the agronomic parameters and chemical composition of the mushrooms harvested.[5,6]

Popularly known as 'sun mushroom', due to their production in the field, exposed to the environmental variables in some regions of Brazil,[7,8] *A. subrufescens* is cultivated in other countries in controlled environments either in plastic greenhouses or chambers where temperature, humidity and CO_2 content are controlled.[9,10]

Sun mushrooms present interesting medicinal properties such as anticancer, host-mediated antitumor activity, immune-stimulatory,

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cytotoxic, antioxidant, antiviral, antibacterial, antidiabetic, antihypertensive, antithrombotic, antianaphylaxis and neuroprotective activities; they can also represent an adjuvant to improve vaccine efficacy and can be used for nutraceutical and cosmeceutical applications.[11,12] Bioactive and antimicrobial compounds present in mushrooms, such as phenolic compounds, ascorbic acid, ergosterol, polysaccharides, carotenoids, steroids, vitamins, terpenes and quinones, have been identified as antioxidants, nutraceuticals and pharmaceuticals.[13,14] Polysaccharides are another class of bioactive compounds biosynthesized by this fungus. Some studies demonstrate the influence of cultivation practices on the production and quantification of β-glucan in mushrooms.[15-18]

Cultivation of mushrooms in the field has some advantages, including lower production costs and high nutritional and bioactive compound content. Nevertheless, production strongly depends on ideal climatic conditions.[19] Field cultivation also involves greater human attention and exposure to environmental conditions. It even has issues related to the health of workers related to the long crouching times required to harvest the mushrooms. In a controlled environment, the pros outweigh the cons: smaller physical space (lower amounts of compost per area) and constant production in all seasons, shorter cultivation cycle, interval control of the flushes, control of pests and diseases, pasteurization of the spent mushroom substrate, adaptation of environmental conditions to the strain used, and less harsh conditions for workers at harvest.[9,20,21]

The biosynthesis of nutritional and bioactive in cultivated edible mushrooms is dependent on the species, stage of development, strain, nutrient substrate and microclimate in the culture space.[6] Hence the aim of the present study was to verify the influence of cultivation process (in the field and under controlled conditions) in the biosynthesis of ergosterol and its bioconversion into ergocalciferol (vitamin D_2) by using commercial strains of *A. subrufescens*. Agronomical parameters are also critically examined.

MATERIALS AND METHODS
Two crop cycles were simultaneously carried out. The first was performed in the field, exposed to environmental variables (solar radiation, wind and rain); the second was conducted under controlled conditions (i.e., temperature, relative humidity and CO_2 partial pressure control). In both cultivation scenarios, four strains and the same compost were used. The casing layer in the field was composed of soil (yellow dystrophic argisol), used as the deposition site for the colonized compost, whereas in the controlled chamber a Dutch commercial peat-based casing was used.

Strains
Four commercial Brazilian strains of *A. subrufescens* were used: ABL 04/49 (isolated from a grower in the region of São José do Rio Preto, GenBank number SP-MW89464.7); ABL CS7 (obtained from the Federal University of Lavras, GenBank number MG-MW200295.2); ABL 18/01 (isolated from a grower in the region of São Paulo, GenBank number SP-MW200293.2) and ABL 19/01 (isolated from a series of crops at CECOG/UNESP, in the city of Dracena, GenBank number SP-MWMW89464.8). The strains were deposited in the collection of the Centro de Estudos em Cogumelos (CECOG) at Universidade Estadual Paulista (UNESP) campus Dracena, SP.

Spawn production
Inocula were prepared following the production steps: selection of mushroom and production of subculture; parent spawn; and finally grain spawn. The grain spawn was produced over sorghum seeds.[22] Briefly, the seeds were boiled at 100 °C for 30 h and then added (0.5 kg wet weight) to polyethylene bags and mixed with limestone (2.0% w/w). The bags were then inoculated and incubated in a dark room at 20 °C for 15 days.

Compost
Compost was prepared by following the traditional two-phase method, lasting 25 days of phase I and 7 days of phase II, for a total of 32 days. The formulation used was composed of 700 kg dry weight *Panicum maximum*, 1200 kg dry weight sugarcane bagasse, 40 kg dry weight soybean, 4 kg urea, 4 kg ammonium sulphate, 8 kg superphosphate and 35 kg limestone. Bulky plant-derived materials (*P. maximum* straw and sugar cane bagasse) were moistened for 6 days and rotated after 3 days. Soybean and chemicals (urea, ammonium sulfate, simple superphosphate and lime) were added after each turning operation throughout the composting phase I, as described in the literature.[23] During phase II, the compost remained for 15 h at 59 $\pm$ 1 °C for pasteurization and 5 days at 47 $\pm$ 2 °C for thermophilic conditioning. The chemical characteristics of the compost at the end of phase II were: N (22 g kg^{-1}), P$_2$O$_5$ (13.8 g kg^{-1}), K$_2$O (34.6 g kg^{-1}), Ca (66 g kg^{-1}), Mg (6.6 g kg^{-1}), S (16.8 g kg^{-1}), Na (3846 mg kg^{-1}), Cu (181 mg kg^{-1}), Fe (1095 mg kg^{-1}), Mn (190 mg kg^{-1}), Zn (190 mg kg^{-1}), C (390 g kg^{-1}), C/N ratio (17:1), organic matter (680 g kg^{-1}) and pH 7.2. Chemical analysis of the substrate was evaluated following the methodology presented by Bell and Ward[24] and Sonneveld and van Elderen.[25]

Inoculation and mycelium run
Inoculation of the compost was conducted in plastic boxes measuring 40 × 50 cm (0.2 m^2, 9 kg fresh compost) in the proportion of 10 g kg^{-1} inoculum relative to the fresh weight (fw) of the compost (90 g spawn). The boxes were incubated for 20 days (until the fungus colonized the entire compost) at 28 $\pm$ 1 °C and 80 $\pm$ 5% humidity in the dark. The colonized compost was then used for the evaluation of both cultivation conditions (field and controlled environment).

Cultivation in the field
Field cultivation in Dracena, SP, at UNESP campus (21° 27′ 33.8″ S and 51° 33′ 18.7″ W) began 20 days before inoculation by opening of the furrows, when calcium carbonate was spread to adjust soil pH to 7.5. The grooves were opened to 50 cm width and 30 cm depth, and were spaced at a distance of 60 cm. The colonized compost (9 kg, w/w) was removed from the plastic boxes, accommodated in the furrow, and then the soil removed was added over the colonized compost as a covering layer (5 cm height). A layer of dry straw, of approximately 50 cm, was added on top of the casing layer to prevent the soil drying (Fig. 1). Once a day, the place was irrigated to maintain a moist microclimate.[19] The harvest phase in the field was 80 days.

Cultivation in the controlled chamber
Cultivation under controlled conditions was performed in a specific mushroom room (Walk-in model CCW-2600, MS Tecnopon, Piracicaba, SP, Brazil), with accurate control of temperature, moisture, and CO_2 content of the environment. Exposure to light was provided by incandescent lamps, which were only lit during
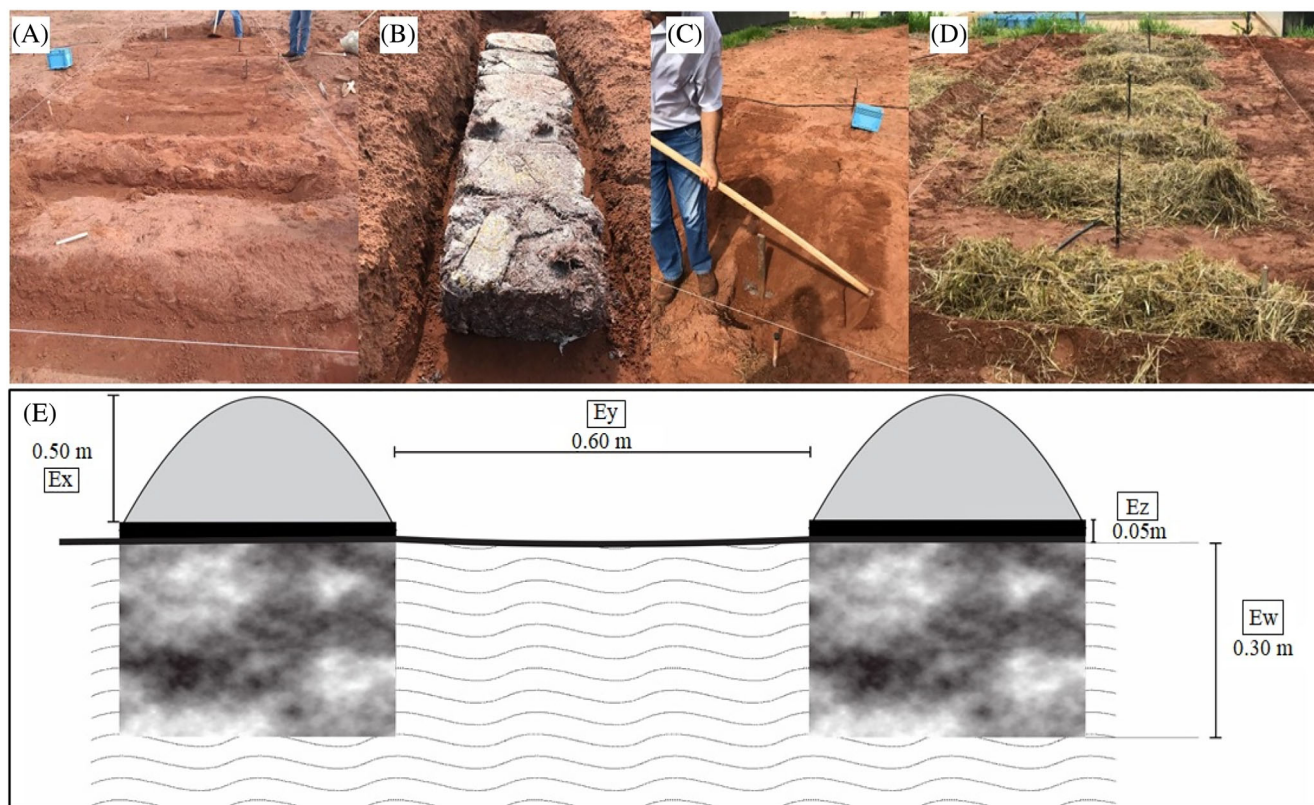
Figure 1. Field production: (A) furrow opening in the soil; (B) addition of the colonized compost in the furrow; (C) covering the compost with the same soil removed; (D) covering the casing layer with straw; and (E) cultivation conduction scheme. Ex, height of straw; Ey, distance between cultivation lines; Ez, height of casing layer; Ew, depth of furrow planting.

harvesting and irrigation of the casing layer. For mushroom production in the controlled environment, the colonized compost was kept inside the plastic boxes (9 kg w/w) and the casing layer was 4 cm in height. A Dutch commercial peat-based casing was used, as reported in the literature,[26,27] with the following characteristics: pH 7.8, 712 g water kg^{-1} substrate, 875 mL L^{-1} total pore space, 4.1 kg water kg^{-1} substrate holding capacity and 271 $\mu S\ cm^{-1}$ electrical conductivity. In the first 10 days, the temperature was maintained at 28°C, then decreased at a rate of 2 °C d^{-1} until 20 °C, remaining for 24 h at this temperature, and then increased by 2 °C d^{-1} until 28 °C.[26] The relative humidity of the environment was maintained at 90%. This regime was maintained to induce four harvest flushes within 57 days of the harvest phase.

Agronomic parameters

The mushrooms were harvested, identified, weighed, counted and dehydrated for agronomic and biochemical evaluation. The following agronomic parameters were evaluated: earliness (days to first harvest expressed as the number of days between inoculation of the compost and harvesting of the first flush); precocity (obtained by dividing the harvest time into two periods where [(yield in the first period/total yield harvested)]; yield [(fw of mushroom/fw compost multiplied) × 100] and expressed as %); biological efficiency [(fw of mushroom/dry weight (dw) of compost) × 100] and expressed as %); production rate [(biological efficiency/total of crop cycle)]; the number of mushrooms (mushroom count in 9 kg substrate, w/w); unitary weight of mushroom [(total fw harvested during the crop/number of mushrooms)].

Chemical analyses (ergosterol and vitamin D_2)

Ergosterol and vitamin D_2 were quantified after extraction following the procedure previously described, with some modifications.[28] Each sample (2.5 g dried mushrooms) was first extracted with 10 mL dimethyl sulfoxide (DMSO) in an ultrasonic bath at 45 °C for 30 min (series LBX V05, Barcelona, Spain), followed by filtration and the addition of 10 mL methanol–water (1:1, v/v) and, finally, filtration and addition of 20 mL hexane using the ultrasonic bath under the same conditions. The solid residues were then centrifuged three times at 4000 × g for 10 min (Centurion K24OR, Chichester, UK), by adding 3 × 20 mL portions of hexane and removing the supernatant between each step. At the end of the extraction, the supernatants were pooled and the solvent was removed using a rotatory evaporator under vacuum at 40 °C in a water bath (Büchi, Flawil, Switzerland). The residue was dissolved in 1 mL methanol and filtered using a Whatman 0.1 μm Nylon filter (Millipore, Billerica, MA, USA). Ergosterol and vitamin D_2 were determined by high-performance liquid chromatography (HPLC) coupled to a UV detector (280 nm), as previously described.[29] Ergosterol (Sigma Aldrich, St Louis, MO, USA) and vitamin D_2 (Acros Organics, Morris Plains, New Jerser, USA) were identified and quantified by comparison with pure chemical standards. Both contents were expressed in mg kg^{-1} dw.

Statistical analysis

Experiments were carried out with four treatments (strains) of *A. subrufescens* under two environmental cultivation conditions. Each treatment had ten replicates with reference to a box containing 9 kg compost. Standard deviations were calculated from

Table 1. Agronomic performance of *Agaricus subrufescens* strains in field cultivation with low technological level

Strain	Earliness (d)	Precocity (%)	Biological efficiency (%)	Production rate (BE% d^{-1})	Yield (%)	Number of mushrooms (u)	Weight of mushroom (g)
ABL 04/49	40.6 ± 0.3	57.3 ± 4.9	40.3 ± 3.6	0.40 ± 0.03	13.75 ± 1.2	26.8 ± 1.9	30.72 ± 1.4
ABL CS7	43.8 ± 0.7	60.7 ± 4.6	61.4 ± 3.1	0.61 ± 0.03	20.95 ± 1.0	36.0 ± 2.5	35.35 ± 1.5
ABL 18/01	43.6 ± 0.3	61.8 ± 7.1	46.7 ± 4.2	0.46 ± 0.04	15.93 ± 1.4	22.0 ± 1.6	43.21 ± 0.6
ABL 19/01	45.6 ± 0.6	69.8 ± 6.0	50.2 ± 5.9	0.50 ± 0.05	17.14 ± 2.0	34.6 ± 4.0	29.73 ± 1.0
Mean	43 ± 2	63 ± 5	50 ± 9	0.49 ± 0.08	17 ± 3	30 ± 7	35 ± 6

Table 2. Agronomic performance of *Agaricus subrufescens* strains in controlled cultivation with high technological level

| Strain	Earliness (d)	Precocity (%)	Biological efficiency (%)	Production rate (BE% d^{-1})	Yield (%)	Number of mushrooms (u)	Weight of mushroom (g)
ABL 04/49	36.8 ± 0.4	63.86 ± 3.49	47.5 ± 1.9	0.61 ± 0.02	16.20 ± 0.68	23.0 ± 3.2	43.27 ± 2.6
ABL CS7	40.5 ± 2.1	71.91 ± 4.27	33.0 ± 3.6	0.42 ± 0.04	11.26 ± 1.24	18.4 ± 1.7	36.31 ± 2.0
ABL 18/01	38.5 ± 0.4	73.12 ± 7.28	50.8 ± 4.5	0.60 ± 0.05	17.33 ± 1.55	26.1 ± 3.2	41.21 ± 2.7
ABL 19/01	37.6 ± 0.3	63.27 ± 4.21	63.6 ± 5.0	0.82 ± 0.06	21.69 ± 1.72	54.5 ± 2.3	23.76 ± 1.5
Mean	39 ± 2	68 ± 5	49 ± 13	0.6 ± 0.2	17 ± 5	31 ± 17	36 ± 9
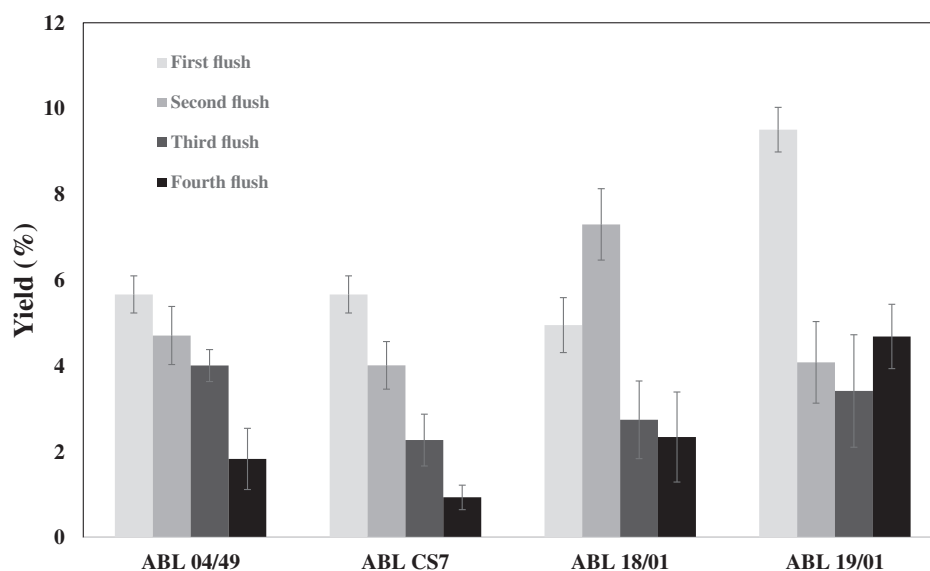
Figure 2. Distribution of yield during the cultivation cycle (four harvest flushes).

Table 3. Ergosterol and vitamin D_2 content in mushrooms cultivated in the field and in protected environment crops

	Ergosterol (mg kg^{-1} dw)			Vitamin D_2 (mg kg^{-1} dw)		
Strains	Field	Controlled	Efficiency decrease (%)	Field	Controlled	Efficiency decrease (%)
ABL 04/49	990 ± 2	839 ± 1	15.3	36.8 ± 0.3	34.1 ± 0.1	7.3
ABL CS7	753 ± 2	716 ± 2	4.9	23.6 ± 0.3	18.6 ± 0.1	21.2
ABL 18/01	971 ± 1	874 ± 7	10.0	19.5 ± 0.1	18.8 ± 0.1	3.6
ABL 19/01	988 ± 8	824 ± 5	16.6	24.0 ± 0.4	20.4 ± 0.2	15.0
Mean	930 ± 12	810 ± 7	12.0	25.9 ± 0.7	22.9 ± 0.7	11.8

Table 4. Stepwise regression analysis for mushroom production parameters and environmental influence of ergosterol and vitamin D_2

Correlation with variable	Equation	R^2 (%)	P	Standard errors of the estimate
Field yield (FY)	FY = 12.0749 − 0.082929 × precociousness + 0.34454 × number of mushrooms	87.7481	0.0429	2.31494
Controlled yield (CY)	CY = 34.0155 − 0.504843 × earliness + 0.238617 × number of mushrooms	98.4911	0.0225	1.31247
Field ergosterol (FE)	FE = 33.9092 + 0.732864 × controlled ergosterol	72.6392	0.0311	6.64177
Field vitamin D_2 (FV)	FV = 0.2989 + 0.983526 × controlled vitamin D_2	93.4810	0.0016	0.17016

results obtained under different conditions, with the presentation data of the media, accompanied by the range of variation verified in each repetition within the treatment. Finally, multiple linear regression models were tested with a forward stepwise regression method of the multiple regression procedure. Differences were considered significant for $P < 0.05$. All statistical analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA).

RESULTS
Cultivation of mushrooms in the field, using ABL CS7 and ABL 19/01 strains, presented better agronomic performance, standing out

due to their high biological efficiency (%), yield (%), production rate (BE% d^{-1}) and number of harvested mushrooms (units). However, by comparing these two strains some interesting properties were observed: ABL CS7 had the highest yield and ABL 19/01 presented higher precocity, indicating a shorter production crop cycle within the total harvest period of 100 days – strikingly different from the controlled environment, which lasted 57 days (Table 1).

In the controlled cultivation, ABL 19/01 presented better agronomic parameters, followed by ABL 04/49 and ABL 18/01, standing out due to their high biological efficiency, yield, production rate and number of harvested mushrooms. Comparison of these three strains indicated that ABL 19/01 had the highest yield and number of mushrooms harvested; nevertheless, it presents a

low weight per unit on average, compared to the ABL 04/49 and ABL 18/01 strains, with a higher weight per unit present. All strains were precocious, with ABL CS7 and ABL 18/01 standing out (Table 2).

The ABL 04/49, ABL CS7 and ABL 19/01 strains showed the highest yield in the first flush, highlighting the significance of ABL 19/01, while the ABL 18/01 showed the highest yield in the second flush (Fig. 2). ABL 19/01 also stood out for its higher yield in the last flush. Cultivation in a controlled environment presents this great advantage concerning the field cultivation, which has daily harvesting with no flush interval of 4–5 days.

Comparing the crops in the field and in a controlled environment, it was found that in the field the time for the first flush was longer (earliness), with mean values of 43 days, compared to 38 days in the controlled environment. The same was observed for precocity, with mean values of 63% and 68.0%, respectively. These results can be explained by the fact that a large part of the yield is concentrated in the first half of the total time of harvest phase. The ABL CS7 strain was extremely dependent on the cultivation environment, being the most productive in the field and less so in the controlled environment. The ABL 19/01 strain in both crops presented mushrooms with low weight.

Regarding ergosterol content in field cultivation, the strains ABL 04/49, 18/01 and 19/01 yielded mushrooms with similar ergosterol content, whereas in a controlled environment all strains had a decrease in ergosterol level, with the CS7 strain presenting the least variation in relation to the field (efficiency decrease of 4.9%). However, this strain remained the one with the lowest ergosterol content. Concerning vitamin D_2 content in mushrooms, the ABL 04/49 strain stood out in both cultivation conditions, showing an even higher amount of vitamin D_2 (34.1 mg kg^{-1}) in mushrooms cultivated in a controlled environment than the other strains cultivated in the field (ABL CS7 23.6 mg kg^{-1}, ABL 18/01–19.5 mg kg^{-1} and ABL 19/01–24.0 mg kg^{-1}) (Table 3).

The agronomic results obtained in field cultivation can be explained through the equation FY, in which yields are negatively correlated with precocity ($r = -0.6412$ and $P = 0.0429$) and positively correlated with the number of mushrooms ($r = 0.9238$ and $P = 0.0085$). In the controlled environment the results can be explained through the equation CY, whose yields are negatively correlated with earliness ($r = -0.7814$ and $P = 0.0664$) and positively correlated with the number of mushrooms ($r = 0.8600$ and $P = 0.0280$) (Table 4).

The efficiency of ergosterol and vitamin D_2 in mushrooms cultivated in the field and controlled environment can be predicted by equations FE and FD. They present positive correlations between the parameters obtained in the different cultivation conditions (ergosterol values of $r = 0.8552$ and $P = 0.0311$; vitamin D_2 values of $r = 0.9668$ and $P = 0.0016$); namely, for all studied strains the increase in the value of ergosterol will not decrease the vitamin D_2 content. This is a useful model that can be applied in any country to compare the efficiency of bioconversion of ergosterol and vitamin D_2 in a controlled environment, in relation to field cultivation.

DISCUSSION

Yield is one of the main parameters considered by the mushroom industry.[30] The highest yield was similar under both conditions in this work, with values of 21% (ABL CS7 strain) in the field and 22% (ABL19/01 strain) in a controlled environment. In this sense, it is possible to observe that the cultivation of *A. subrufescens* can be carried out by small producers/family farms up to large mushroom industries.

Agaricus subrufescens can be cultivated during the summer, usually an unfavorable season for the production of *A. bisporus*, using the same physical facilities and the same compost that would be used for the production of button mushrooms.[27] This culture rotation is energy-saving since the sun mushroom requires a higher temperature for cultivation and can increase the producer's income due to the commercial value of this mushroom.[31]

Moreover, it is possible to commercially produce *A. subrufescens* in controlled environments with high biological efficiency and short crop time (83 days), which allows four harvesting cycles per year in the same cultivation chamber.[26] Similar results were obtained by Llarena-Hernández *et al.*[27] with the wild strains CA 487 (24.4%) and 438-A (26.2%), during their 85-day growth cycle.

The mushrooms obtained in the field are mycochemical superior compared to those obtained in a protected environment; however, the quality screening of mushroom strains is a crucial factor that allows us to find strains that can be used, generating less yield losses of vitamin D_2, as is the case for ABL 04/49 cultivated in a controlled environment.

Vitamin D can be available as ergocalciferol (vitamin D_2) obtained from yeasts and mushrooms, and cholecalciferol (vitamin D_3) available in animal products and produced by the skin after sun exposure.[32,33] Vitamin D_2 can be obtained from UV-irradiated yeasts and mushrooms due to photochemical cleavage of the ergosterol B ring, which forms the intermediate pre-vitamin D_2 and then ergocalciferol by thermal rearrangement.[34,35] Some wild mushrooms contain vitamin D_2 because they are naturally exposed to UV light, whereas cultivated species contain more ergosterol.[36]

The ergosterol values reported in this work – between 716 and 990 mg kg^{-1} mushrooms – are similar to those observed by Rózsa *et al.*[6] between 710 and 957 mg kg^{-1}, and thus the nutrient supplementation of compost increases the vitamin content of fruit bodies of *A. subrufescens*. These values are, in general, lower than those observed in *A. bisporus* and other species.[6,37,38]

In the present study, the results obtained demonstrated that in both cultivations carried out in the field and in a controlled environment the values of ergosterol and vitamin D_2 were higher in mushrooms grown in the field, with means varying from 4.9% to 16.6% for ergosterol content and from 3.6% to 21.2% for vitamin D_2 content in a controlled environment. The strain that presented superior mycochemical quality was ABL 04/49, followed by the 19/01 strain.

Vitamin D_2 deficiency has been recognized as pandemic and associated with numerous diseases such as cancer, cardiovascular diseases, obesity, diabetes, rheumatoid arthritis, osteoporosis and rickets in children, leading to bone malformation.[39] The importance of vitamin D in the human diet is widely recognized, promoting calcium absorption and maintaining adequate serum calcium and phosphate concentrations.[40] Furthermore, it supports the immune system and prevents several illnesses.[41] Therefore, the environmental conditions established in this work are promising and allow the enrichment of the matrix under study, which stands out for being a promising source of bioactive compounds for human consumption and for the production of pharmaceutical preparations and food supplements.

CONCLUSIONS

Remarkable differences have been observed in agronomic parameters and in the chemical distribution of ergosterol and vitamin D_2

content, depending on the strain used and the growing environment. Hence, productively speaking, ABL CS7 and ABL 19/01 strains showed the best agronomic parameters in the field and in the controlled environment, respectively. The ABL 04/49 strain showed the highest ergosterol and vitamin D_2 content in both growing environments.

In recent years, various research studies have tackled the adaptability of *A. subrufescens* to commercial cultivation in the main areas of production of edible mushrooms (substrates, casings, strains, growing cycle management, etc.). The novelty of this work lies in presenting an integrated study concerning productivity and the biosynthesis of ergosterol and vitamin D_2 by different strains of mushrooms growing under different environmental cultivation conditions. Hence this study helps predict behavior and encourages the commercial cultivation of the sun mushroom, a promising source of bioactive compounds for human consumption, for the production of pharmaceutical preparations and dietary supplements.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Wagner GV Junior: investigation, writing and original draft; Rossana VC Cardoso: data collection; Ângela Fernandes: investigation and writing; Isabel CFR Ferreira: formal analysis; Lillian Barros: formal analysis and design; Arturo Pardo-Giménez: conceptualization and supervision; Douglas MM Soares: investigation and writing; Cassius V Stevani: investigation and writing; Diego C Zied: supervision, review and editing.

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