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Induction of low sporulating-UV mutant of oyster mushroom with high content of vitamin D_2.

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Abstract

Oyster mushrooms comprise some most popular edible mushrooms due to their favorable organoleptic and medicinal properties, vigorous growth and undemanding cultivation conditions. One of the major problems in cultivating oyster mushrooms for workers is the abundant production of spores. The inhalation of these spores induces allergic responses. Furthermore, vitamin D deficiency is an ever-increasing problem in human nutrition and health. The present study was initiated to use ultra violet irradiation (UV) to obtain a new mutant of *Pleurotus columbinus* with improved characters and to solve the previous problems.

Mycelial plugs of the actively growing parent were exposed to UV-C for different periods from ten to thirty minutes. Twenty minutes irradiation period showed the best mycelial growth (R20) and was selected for further evaluations. The fresh fruiting bodies of mutant R20 had lower gills number by 32%, lower strigmata by half and reduced spore content by 72% per each cm^3 compared to parent (R0). Moreover, the fruiting bodies of R20 posses higher ability for vitamin D_2 production after UV-C irradiation for different periods. The best vitamin D_2 production was achieved in R20 after 30 minutes exposure (121.95 µg/g fresh mushrooms) compared to parent (60.87 µg/g fresh mushrooms). These new acquired characters of the mutant R20 make it better for commercial production and more attractive for human consumption.

Keywords: *Pleurotus columbinus*; Ultra violet; Irradiation; Mushroom cultivation; vitamin-D_2

Introduction

Oyster mushrooms belong to the genus *Pleurotus,* considered as an ideal food with delicious taste and high nutritional value. They are the second most important mushrooms comprise 30% of total world production of cultivated mushrooms. It can be cultivated on log and a wide variety of agro forestry by-products, weeds and wastes for the production of food, feed, enzymes and medicinal compounds, or for waste degradation and detoxification (Gregori *et al*., 2007 and Awadalla *et al*., 2008). The fruiting bodies of oyster mushroom are gymnocarpous, i.e. spore discharge begins early and continues until the sporophores are harvested into the growimg atmosphere which comprises one of the major problems in cultivating oyster mushrooms. The inhalation of these spores induces allergic

responses like farmer's lung disease and hay fever with symptoms similar to' extrinsic allergic alveolitis' (EAA) (Daba *et al*., 2008). Severity of allergic response may vary from person to person like general fatigue, mild headache, cough, and mild difficulty in breathing, pain in the limbs, etc. (Kurup *et al*.,1987). The spores may also be a source of pollution, which may include new genotypes likely to attack wood or trees. Also, the spores settle on fruit bodies, germinate and lead to the formation of a white, velvety film, which gives an unpleasant appearance to the mushroom during commercialization.

Vitamin D is a fat-soluble vitamin commonly occurring in the D_2 (ergocalciferol) or D_3 (cholecalciferol) forms. Vitamin D_2 is synthesized from the fungal sterol ergosterol through exposure to ultraviolet light while vitamin D_3 is synthesized, in humans, through skin exposure to ultraviolet light and subsequent conversion of cholesterol (Jasinghe and Perera, 2006). Vitamin D plays a vital role in bone mineralization by promoting calcium absorption, and supporting the immune system as well. An adequate vitamin D level is necessary to prevent different kind of illnesses, such as heart diseases, obesity, diabetes, arthritis, hypertension, inflammatory bowel disease, multiple sclerosis, mental illness, stroke, chronic pain etc. (Cannell *et al*., 2008). Moreover, vitamin D has recently been linked to a significantly reduced risk of breast cancer, colon cancer, prostate cancer (Lappe *et al*., 2007), autoimmune disease, and cardiovascular disease (Wang *et al*., 2008). Vitamin D deficiency is an ever-increasing problem in human nutrition and health all over the world. Hence, the introduction of an alternative dietary source of vitamin D would most likely be helpful in protecting this population from such a deficiency. Although, higher amount of vitamin D_2 is present in wild grown mushrooms, than in cultivated species as cultivated mushrooms are grown in the dark, where the absence of UV radiation results a lower level of vitamin D_2, but they contain more ergosterol than wild grown mushroom species (Teichmann *et al*., 2007 and Jasinghe *et al*., 2007). Number of studies proved that ergosterol content of post-harvest mushrooms can be converted into vitamin D_2 and its concentration can be enhanced up to nine folds by applying artificial UV irradiation on a certain wavelength (Jasinghe, 2005).

Research was undertaken to solve the spore problem through the development of spore less or low-sporulating strain of *Pleurotus* with enhanced vitamin D_2 content using UV-C light irradiation which would be preferable for commercial production.

Materials & Methods

1. Mushroom species:

Pure culture of the cultivated oyster mushroom, *P. columbinus* has been used. It was supplied from consultative commet company of mushroom cultivation, Giza -Egypt (CCCM).

2. Ultra violet irradiation

Seven-days-old actively growing culture of *P. columbinus* on PDA medium (200 g potato extract, 20 g glucose, 20 g agar per one liter of distilled water) with uncovered plate was exposed directly to UV-C lamp (15 watt, λ= 250 nm, Philips, Holland) at 60 cm distance at time intervals (0, 10, 20 and 30) min. One cm diameter discs were cut randomly with a sterile cork borer from the margins of the irradiated cultures and transferred to 12 cm sterilized petri dishes with PDA medium in triplicates then incubated at 28°C under dark conditions. The diameter of growth was measured after 3 and 5 days of incubation (Osman *et al*., 2007). The irradiated fungus was subcultured several times on PDA media and the differences in mycelial diameter and cultures characterization were observed.

3. Mushroom cultivation

Mushroom cultivation was carried out according to El-Fallal *et al*. (2009) in the mushroom cultivation house belonging to Botany Department, Faculty of Science, Damietta University.

Spawn was prepared using sorghum grains which inoculated with fully grown PDA cultures, then incubated at 28°C for two weeks in the dark. Substrate was prepared using chopped rice straw that soaked overnight in water then, pasteurized by steaming and filled in plastic bags inside sterile mushroom house. After spawning and fully colonization of the substrate with the white mycelial growth; small holes were made around the bag to provide aeration and relative humidity was maintained at 70-85% by using foggers and watering the floor twice a day. The fruiting bodies were harvested daily then used for comparison of growth and biochemical analysis.

4. Sporophores characters

Visual spore production was observed by spore print; the fruiting bodies were placed on the dry surface with the gills facing downwards and left over night for spore deposition. Spore print colour was noted (Pandey and Ravishankar, 2010).

Sporophores characters were observed microscopically by gill sectioning, gills number and spore count (1cm of each fruiting body was suspended individually in known volume of sterile distilled water. The spore number was counted using haemocytometer. 10 µl of spore suspension were placed on the haemocytometer; the spores were counted in all 25 squares. The total number of spores was calculated using the following formula: Spores/cm=Total cell count in 25 squares x 10,000x dilution). Size of spores was measured using ocular & stage micrometer (Ravishankar *et al.*, 2006).

5. Induction of vitamin D_2

The fresh fruiting bodies of *P. columbinus* (R0) and its mutant (R20) were used directly after harvest according to the method of Teichmann *et al*. (2007). 10 g samples were cut into $1cm^3$ cubes and

spread as monolayer on aluminum trays then exposed to UV-C for 0, 15, 30, 45, 60 min at 25°C in an irradiation cabinet. The samples were placed with their gills facing the lamp on 20 cm distance away from it. Samples were air dried under cold conditions (at 5°C) directly after treatment and ground into powder then stored at -20 °C.

(1-2 g) dried mushroom samples were extracted twice with 10 ml of petroleum and diethyl ether (1:2 V/V) mixture for 24 h with shaking under cold conditions (5 °C). Then extracts were separated and evaporated under cold conditions. The residues obtained were dissolved in150 µl of a chloroform /methanol mixture (1:3 V/V). The solution was then filtered through 0.45 µm nylon membrane syringe filters (Mattila *et al*., 2001).

The identification and quantification of vitamin D_2 were carried out by High-Pressure Liquid Chromatography (HPLC) using Brinary LC Pump-250 (Perkin Elmer) system made in USA at the following conditions: Column: 5 µm C18 column (250 × 2.00 mm), Mobile Phase: water/methanol (5:95 V/V), Flow Rate: 1.2 ml/min, Detector: The detection wavelength was set to 265 nm and The samples run time: 40 min. Standard vitamin D_2 (100 mg) sample was dissolved in 2 ml of $CHCl_3$/MeOH (1:1 V/V) to give a standard solution containing 50 mg/ml of vitamin D_2 for the HPLC analysis (figure 2.7) (Koyyalamudi *et al*., 2009). An amount of 20 µl was then injected into the analytical reversed-phase HPLC system. Vitamin D_2 was quantified by comparing the peak area to that of the internal standard. For checking the stability of the retention times, the standard solution was injected 2–3times (Mattila *et al*., 2001).

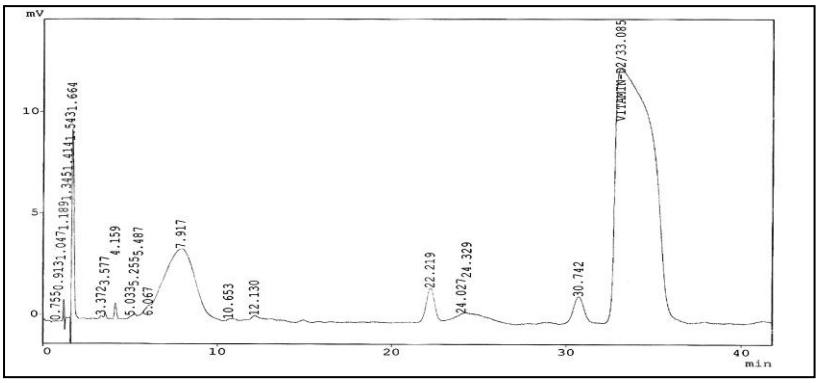

Figure 2.7: High performance liquid chromatogram of standard vitamin D_2

5. Statistical analysis

Data generated were analyzed using one SPSS program (v.11). The level of significance was set at ($p < 0.05$). Means were compared using paired samples t-test.

Results

The most obvious observation was the significant increase in the radial growth of three treatments of *P. columbinus* compared to its parent, but the mutant of 20 min UV exposure (R20) showed the highest increase in colony diameter compared to those exposed to 10 min, 30 min or the untreated parent (R0).

The spore content of mutant R20 fruiting bodies was reduced significantly by 72% in comparison to untreated parent (R0). However the size of spores in case of mutant R20 is much bigger than R0 as presented in table 1. Sporophores of *P. columbinus* mutant R20 had very faint spore density visually in spore print compared to highly dense spore of the parent R0 (plate 1A). Another characteristic change brought about due to mutation was the reduction in the number of gills/cm^2 in the low sporulated mutant R20. The number of gills was reduced by 32% comparing to the control per each cm^3 as represented in table 1 and plate 1B.

The gills section of the sporophores of the control R0 showed basidia possessing four sterigmata and four basidiospores with moderate size. On the opposite side, the gills section of R20 sporophores showed very few attached spores. However, in some places the sterigmata could be seen as only two large sterigmata without attached spores. Hence this mutant was designated as a low sporulated mutant (plate 1C). The fruiting bodies of the two crops (during simmer & autumn seasons) showed nearly the same results for spores and gills of both R20 and R0.

Table 3.8. **Comparison between gills and spores of both *P. columbinus* (R0) and its mutant (R20).**

Mushroom	**No. of gills/cm^3**	**No. of spores/cm^3**	**Spore size(mm)**	
			Length	**Diameter**
R0	15.00-19.00	$160x10^4$ -$176x10^4$	$99x10^{-4}$-$132x10^{-4}$	$50x10^{-4}$ -$66x10^{-4}$
R20	9.00-13.00***	$32x10^4$ -$48x10^4$ **	$165x10^{-4}$-$198x10^{-4}$	$66x10^{-4}$ -$99x10^{-4}$

All values are mean ±SEM of 6 replicate. (**) means significant difference at $P < 0.01$, (***) means significant difference at $P < 0.001$.

The results of HPLC analysis for vitamin D_2 content of *P. columbinus* (R0) and its mutant (R20) are presented as µg per g fresh mushroom in figure 1. Results indicate that vitamin D_2 was absent in fresh sliced mushroom for both R20 mutant and its parent R0 before UV-C treatment. Its content in both mushrooms increased with increasing the post harvest UV-C exposure time at 30 min then decreased at 60 min exposure period. It is clear that Vitamin D_2 content of the mutant R20 was twice the value of its parent R0 at both 30 and 60 min exposure periods. Vitamin D_2 was studied in R20 to detect the best UV exposure period for optimal vitamin production. The dose of 30 min was found to be the optimal exposure period which gave the highest production of vitamin D_2 in R20. Vitamin D_2

production of 15 min dose was higher than 45 and 60 min doses (figure 2). HPLC of vitamin D_2 estimation of *P. columbinus* (R0) and its mutant (R20) before and after UV treatment were presented in figures 3 & 4.

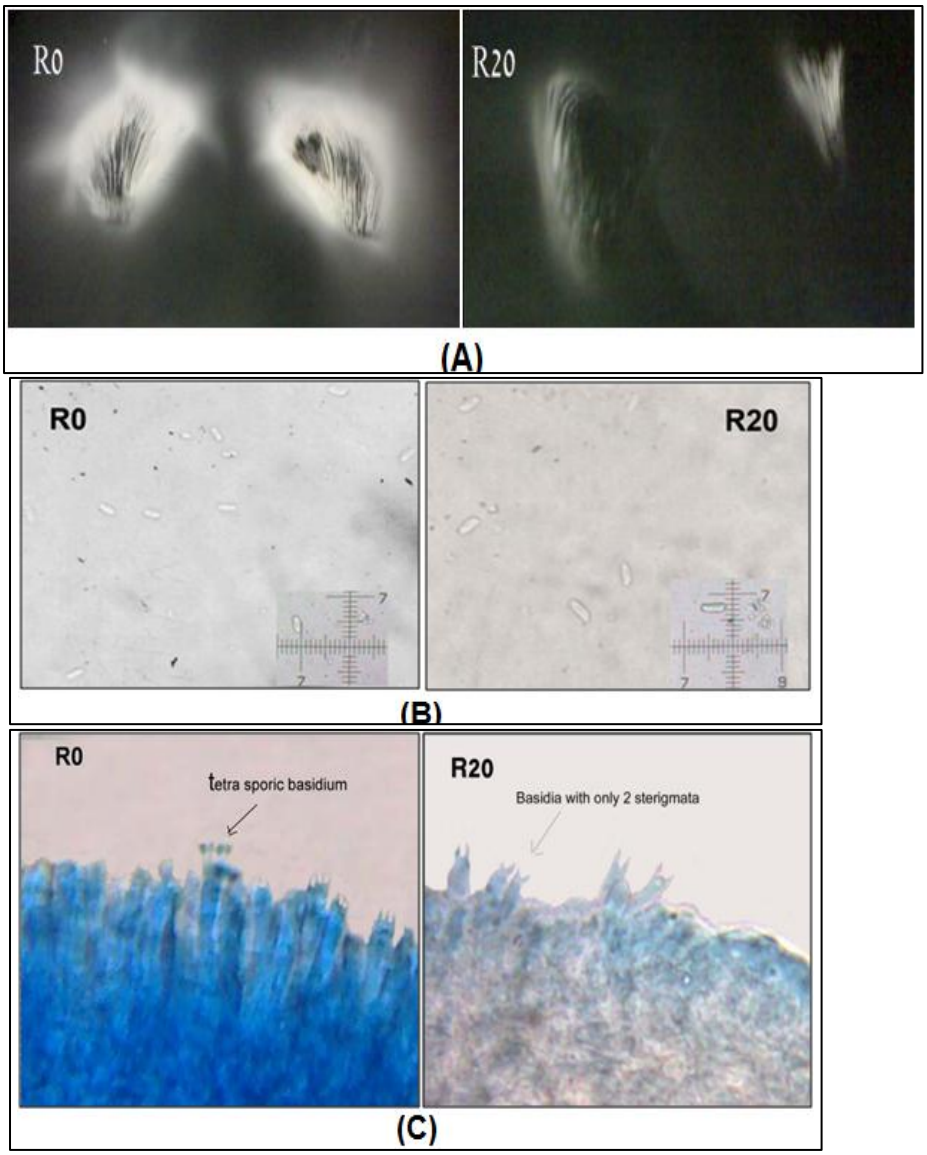

Plate 1: Sporophore characters of *P. columbinus* (R0) and its mutant (R20), (A): Spore print, (B): Spore count using haemocytometer and spore size using ocular micrometer, (C): Gills section showing basidia and strigmata.

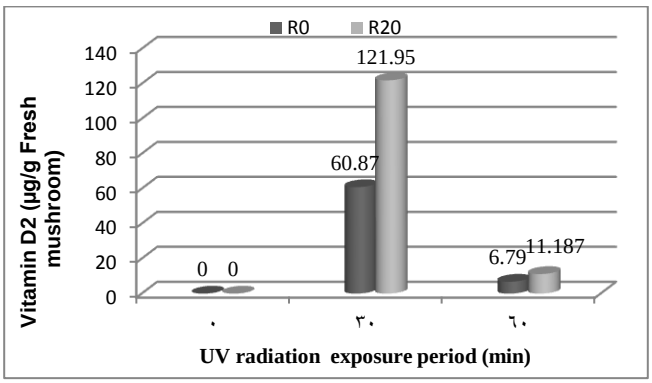

Figure 1: Vitamin D_2 concentrations in *P. columbinus* (R0) and its mutant (R20) mushrooms that treated with UV-C for different periods.

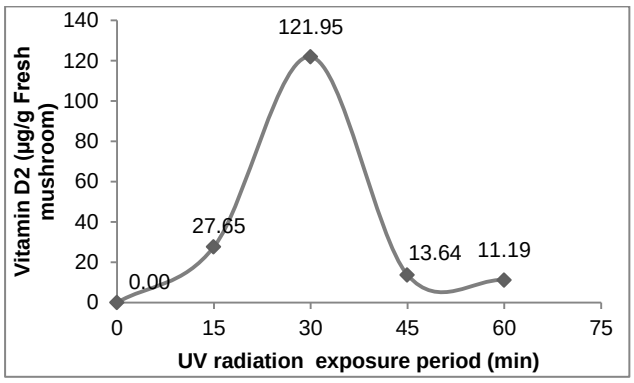

Figure 2: Vitamin D_2 concentrations in both *P. columbinus* mutant (R20) mushrooms that treated with UV-C for different periods.

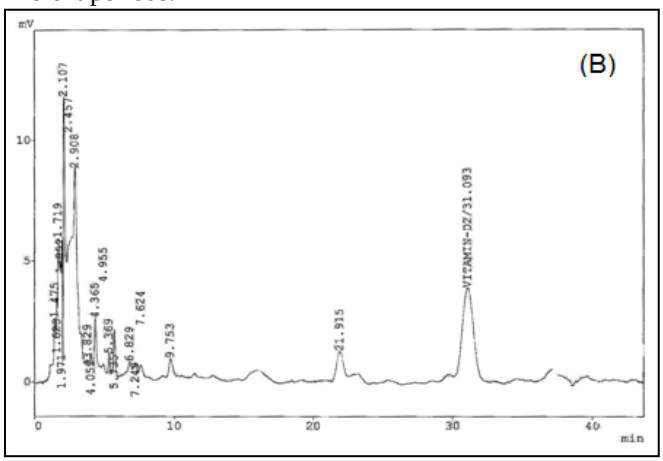

Figure 3: HPLC chromatogram of vitamin D_2 of *P. columbinus* (R0) UV-C treated for 30 min.

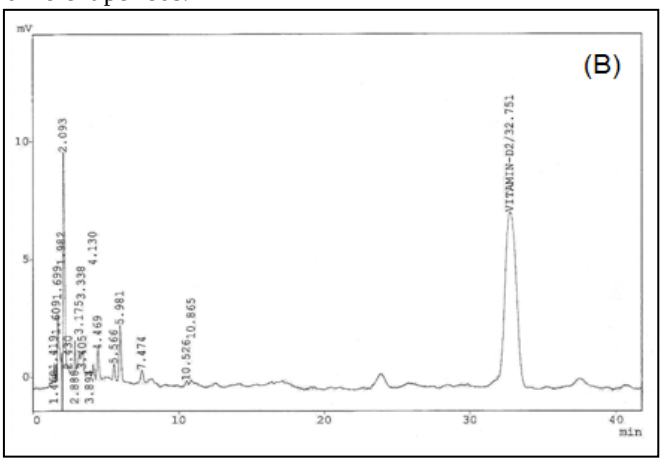

Figure 4: HPLC chromatogram of vitamin D_2 of *P. columbinus* mutant (R20) UV-C treated for 30 min.

Discussion

Oyster mushroom (*Pleurotus*) is an edible mushroom that gained popularity lately due to its nutritional values and ease of cultivation. Oyster mushroom cultivation can be considered environmental friendly and it has increased tremendously worldwide during the last decades. In present study, UV-C rays was applied to mycelia culture grown on PDA agar media to different periods as it was the effective method for radiating mushroom according to Osman *et al*. (2007) and Djajanegara and Harsoyo (2009), by using this method the radiating ions are evenly distributed much easier to reach the cells of the mycelia. Furthermore, post radiation observation on the mushroom morphology is easily observed. Fortunately, the obtained three treatments of *P. columbinus* proved faster and higher mycelial growth over their parent, twenty minutes irradiation period proved to be the best in increasing mycelial growth. These differences in the three new strains were stable across repeating subcultures. Moreover, R20 mutant seems to be more efficient in fruiting bodies production, better growth performance, earlier

flushing and higher yield. There were no differences in morphological characters in term of shape and odor with a little lighter color than parent. Furthermore, it was more tolerant to higher temperatures during summer season as previously mentioned in El- Fallal *et al*. (2013). UV-C may be resulted in some inherited changes in the DNA which lead to a permanent mutation which were assessed by RAPD- PCR results using specific basidiomycetes pimers as previously mentioned in El-Gharabawy (2012).

In the present study, UV mutation resulted in high reduction of R20 spore production which detected by the faint spore print, lower gills, less spore count and reduced strigmata than parent. This is an interesting observation as such low sporulated mutants may solve the previous problem of workers respiratory allergies and it would be safe and suitable for commercial production. The production of sporeless strains in the oyster mushroom has been reported in *P. florida*, *P. pulmonarius* and *P. ostreatus* by earlier workers (Imbernon and Labarere, 1989 and Baars *et al*., 2000), and in other mushrooms (Verma *et al.,* 2000), while the production of a low sporulated UV mutant in *P. sajor-caju* was recorded by Ravishankar *et al*. (2006) and Pandey and Ravishankar (2010). In support, UV mutants of *P. eous* (U6 and U10) produced low sporulated fruiting bodies as revealed by their spore prints (Ramasamy and Muthukumarasamy, 2012). However, the present investigation is the first report of the production of a low sporulated mutant in *P. columbinus.*

Vitamin D is now known to have many beneficial clinical applications. Deficiency of vitamin D among the world population is dramatically increasing. Vitamin D_2 could be provided from mushrooms, and it has some remarkable advantages over vitamin D3. It is more effective for bone mineralization and less toxic compared with vitamin D3. In the present study vitamin D2 was not detectable in mushroom of both *P. columbinus* and its mutant R20 without UV-C treatment. In support, vitamin D_2 was almost totally absent in cultivated mushrooms including *Pleurotus*, although they were found to be a very rich source of ergosterol. On the other hand, wild mushrooms contained high concentrations of this vitamin due to sunlight exposure and UV irradiation which converts the ergosterol to ergocalciferol (vitamin D_2) increasing its content in mushroom (Mattilia *et al*., 2001; Jasinghe, 2005). The increase in vitamin D_2 concentrations in µg/g of dry mushroom of both R0 and R20 was dependent on time of post harvest exposure to UV-C irradiation. The results of our experimental studies showed a great conversion of ergosterol to vitamin during UV treatment of mushroom. This conversion increased linearly with time till 30 min (60.87 & 121.95 µg/g fresh mushroom for R0 and R20 respectively) at which all ergosterol content was converted and the highest vitamin D_2 was generated, then it decreased till 60 min. Fortunately, R20 mushroom seems to be more

efficient in vitamin D_2 production. It had higher vitamin D_2 content than R0 in all UV doses especially 30 min dose at which vitamin content of R20 was twice R0 content.

The observed decrease in vitamin content in both R20 and R0 at longer periods of UV treatment (45 & 60 min) may be attributed to irreversible over-irradiation products which may result in photo-degradation of vitamin D_2 as reported by Webb *et al*. (1989) and Jasinghe and Perera (2006) . According to the previous studies, moisture content of mushrooms plays an important role in this conversion of ergosterol to vitamin D_2. The optimum moisture content of mushrooms for the conversion of ergosterol to vitamin D_2 was found to be around 70% - 80% (Jasinghe *et al*., 2007; Jasinghe, 2005). The increase of vitamin D_2 content in R20 over R0 may be due to higher ergosterol content of R20 mushroom which resulted in a higher conversion to vitamin D_2. Additionally, the moisture content of R20 (74%) is more suitable rather than its parent (86.25%) for the conversion of ergosterol to vitamin D_2. In both cases it's an evident that UV mutation was an effective tool in enhancing the vitamin D_2 production by *P. columbines* (El-Gharabawy, 2012).

Our results for vitamin D_2 agree with Gyôrfi *et al*. (2011), who found that values of vitamin D_2 in cultivated oyster mushroom increased gradually with increasing the UV-C exposure time till 40 min then decreased with longer periods of exposure till 90 min. However, their results were much lower than results of our investigation. This decrease maybe attributed to pre harvest UV treatment not post harvest. The value of vitamin D_2 of R20 was extremely higher than that reported for UV-C treated *Agaricus bisporus* by Teichmann *et al*. (2007) (10.14 µg/ g dry mushroom after 2h of UV-C treatment) and by koyyalamudi *et al*. (2009) who used a wide range of exposure time (from 0 to 60 min). Our results for R20 were higher in vitamin D_2 content than that of different types of mushrooms including oyster mushroom which was subjected to 2 h of UV-A irradiation with their gills facing the source of irradiation, but it was close to the results of UV-C irradiation of oyster mushroom at the same conditions (Jasinghe, 2005).

In fact, the results of this study could be applicable to fresh mushroom industry in order to add more nutritional value to fresh or dried mushrooms with an appropriate dose of radiation. Even though vitamin D_2 is a fat soluble vitamin, that from irradiated mushroom powder could be incorporated with food products without a fat base. An interesting point was the use of irradiated mushroom powder as an additive of vitamin D_2 since the incorporation of irradiated mushroom powder would not change the caloric value or fat composition of the product significantly. Present study clearly showed that, irradiated mushroom powder of R20 could be used in pharmaceutical industry to develop vitamin D_2 drugs that used in eliminating vitamin D deficiency from the effected population.

In conclusion, UV-C irradiation of oyster mushroom *Pleurotus columbinus* was successful for yielding new low sporulated mutant (R20) with higher vitamin D_2 concentrations. These new characters which R20 gained make it better for commercial production and more attractive for human consumption.

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الملخص العربى

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أميرة على الفلال- أحمد قاسم عبد الصمد السيد-هدى محمد ابراهيم الغرباوى

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تعتبر فطريات عيش الغراب المحارى هى الأكثر شعبية بين فطريات عيش الغراب الصالحة للأكل بسبب خصائصها العضوية والطبية المفضلة، والنمو النشط وظروف الزراعة البسيطة. و من المشاكل الرئيسية التى تواجه العاملين فى زراعة فطر عيش الغراب المحارى هي إنتاج جراثيم غزيرة. استنشاق مثل هذه الجراثيم يؤدى إلي حدوث حساسية. علاوة على ذلك، نقص فيتامين د يمثل مشكلة متزايدة فى التغذية والصحة البشرية. فقد استهدفت هذه الدراسة البحثية إستخدام الأشعة فوق البنفسجية للحصول على طفرة جديدة من فطر البلوروتس كولمبينس ذات خصائص أفضل لحل المشاكل السابقة.

تم تعريض الخيوط الفطرية النشطة النمو إلى الأشعة فوق البنفسجية سى لفترات مختلفة من (١٠إلى ٢٠) دقيقة. أظهرت جرعة التشعيع ٢٠ دقيقة أفضل نمو (ر٢٠) وتم اختيارها للاختبارات الأخرى. كما احتوت الأجسام الثمرية للطفرة ر٢٠ على عدد أقل فى الخياشيم بنسبة ٣٢٪، واختزل عدد الذنيبات الي النصف و انخفض المحتوى الجرثومى بنسبة ٧٢٪ في كل سم^{3} بالمقارنة مع الأصل (ر٠). فضلا عن ذلك فان الأجسام الثمرية للطفرة ر٢٠ تمتلك قدرة أعلى لإنتاج فيتامين د٢ بعد التشعيع بالأشعة فوق البنفسجية – سى لفترات مختلفة وتحقق افضل إنتاج لفيتامين د٢ عند تعرض الجسام الثمرية للأشعة فوق البنفسجية لمدة ٣٠ دقيقة (١٢١.٩٥ ميكروجم/جم عيش غراب طازج) مقارنة بالأصل (٦٠.٨٧ ميكروجم/ جم عيش غراب طازج) . هذه الخصائص الجديدة التى اكتسبتها الطفرة ر٢٠ جعلتها أفضل للإنتاج التجارى وأكثر جاذبية للإستهلاك البشري

الكلمات الدالة: بلوروتس كولمبينس-الأشعة فوق البنفسجية- تشعيع –زراعة عيش الغراب- فيتامين د٢