Rev Iberoam Micol. 2018;**35(2)**:103–109

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Original article

Use of lignocellulosic wastes of pecan (*Carya illinoinensis*) in the cultivation of *Ganoderma lucidum*

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ARTICLE INFO

Article history:
Received 6 February 2017
Accepted 4 September 2017
Available online 3 May 2018

Keywords:
Carya illinoinensis
Lignocellulosic waste
Ganoderma lucidum
Biological efficiency
Mushroom production

ABSTRACT

Background: The wastes of pecan nut (*Carya illinoinensis* (Wangenh.) K. Koch) production are increasing worldwide and have high concentrations of tannins and phenols.
Aims: To study the biodegradation of lignocellulosic wastes of pecan used as solid substrate for the cultivation of the white-rot fungus *Ganoderma lucidum* (Curtis) P. Karst.
Methods: Six formulations of pecan wastes were used as solid substrate: pecan shells (PS100), pecan pericarp (PP100), pecan wood-chips (PB100), and the combinations PS50 + PP50, PB50 + PS50 and PB50 + PP50. The substrates were inoculated with a wild strain of *G. lucidum* collected in the Iberian Peninsula. The biodegradation capability of *G. lucidum* was estimated by using the mycelial growth rate, the biological efficiency, the production and the dry biological efficiency.
Results: Notably, all solid substrates were suitable for *G. lucidum* growth and mushroom yield. The best performance in mushroom yield was obtained with PB100 (55.4% BE), followed by PB50 + PP50 (31.7% BE) and PB50 + PS50 (25.4% BE). The mushroom yield in the substrates containing pecan wood-chips (PB) was significantly higher.
Conclusions: Our study is leading the way in attempting the cultivation of *G. lucidum* on lignocellulosic pecan waste. These results show an environmentally friendly alternative that increases the benefits for the global pecan industry, especially in rural areas, and transforms biomass into mushrooms with nutraceutical properties and biotechnological applications.

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Utilización de residuos lignocelulósicos de la pacana (*Carya illinoinensis*) para el cultivo del hongo *Ganoderma lucidum*

Palabras clave:
Carya illinoinensis
Residuos lignocelulósicos
Ganoderma lucidum
Eficiencia biológica
Producción de carpóforos

RESUMEN

Antecedentes: Los residuos de la producción de pacana (*Carya illinoinensis* [Wangenh.] K. Koch) se distribuyen por todo el mundo y poseen elevadas concentraciones de taninos y fenoles.
Objetivos: Estudiar la biodegradación de los residuos lignocelulósicos de la pacana usados como sustrato sólido para el cultivo de *Ganoderma lucidum* (Curtis) P. Karst.
Métodos: Se utilizaron seis formulaciones de sustratos sólidos a partir de los residuos: cáscara de la nuez (PS100), pericarpio de la nuez (PP100), astillas de ramas de poda (PB100) y las combinaciones PS50 + PP50, PB50 + PS50 y PB50 + PP50. Los sustratos se inocularon con las hifas de una cepa silvestre de *G. lucidum* procedente de la península ibérica. La capacidad de biodegradación de *G. lucidum* se estimó mediante el ratio de crecimiento micelial, la eficiencia biológica, la producción de carpóforos y la eficiencia biológica en seco.

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https://doi.org/10.1016/j.riam.2017.09.005
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Resultados: Notablemente, todos los sustratos sólidos utilizados resultaron adecuados para ser colonizados por *G. lucidum* y producir carpóforos. Los mejores rendimientos en cultivo se obtuvieron con la formulación PB100 (55,4% BE), seguida por PB50 + PP50 (31,7% BE) y PB50 + PS50 (25,4% BE). La producción de carpóforos en sustratos con astillas de ramas del árbol (PB) fue considerablemente más elevada que en aquellos que no contenían este residuo.
Conclusiones: Este estudio muestra la posibilidad de cultivar *G. lucidum* sobre residuos lignocelulósicos de pacana. Los resultados obtenidos sugieren una alternativa respetuosa con el medio ambiente para el incremento de los beneficios en la industria de la pacana a nivel internacional, especialmente en zonas rurales, al convertir biomasa en la producción de un hongo de interés nutracéutico y con aplicaciones biotecnológicas.
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The cultivation of *Carya illinoinensis* (Wangenh.) K. Koch is increasing worldwide (i.e. United States, Mexico, Australia, South Africa, Brazil, Argentina, Chile, Egypt, Israel, Spain, etc.).[30,41] Pecan nut production is valued at over USD 433 million in the United States (USDA, 2008). In 2012, the worldwide production of nuts rose to 7 million tons, of which 187,000 tons were produced in Europe and 13% were produced in the Iberian Peninsula.[10] The direct disposal of waste from pecan nut yields is often neglected,[38] which represents an important loss of biomass and it is a major cause of environmental pollution. In fact, the pecan nutshell processing generates an important amount of waste in the form of shells (40–50%).[30,35] The waste products of pecan are rich carbon sources[1] and have high concentrations of tannins and phenols.[7,23] The composition of lignocellulosic wastes of pecan, in terms of percentage of cellulose, hemicellulose, lignin and ash in the shells, are 5.6, 3.8, 70 and 5.85%, respectively,[1] in the pericarp are 30, 26, 41 and 1.7%,[13] and in the pecan branches are 38.7, 30.2, 23.3 and 0.4%, respectively.[28]

The ability of *Ganoderma lucidum* to decompose lignocellulosic wastes has been extensively studied.[18,45] So far, there have been no studies to find out whether these white-rot fungi (WRF) can utilize lignocellulosic pecan waste. The mushroom *G. lucidum*, known as *reishi* (in Japan) and *lingzhi* (in China), has been used for centuries in oriental traditional medicine and it is one of the most important and widely distributed WRF in the world. *G. lucidum* has been linked to nutraceutical mushrooms. In clinical trials with *G. lucidum*, hepatoprotective efficacy,[5] control in type 2 diabetes[42] and protection against lung cancer[37] were observed. Additionally, biotechnological applications of the fungus, for example as a producer of enzymes that decolorize the synthetic dyes from effluents,[16,24] or in oleo-chemical and biotechnological industries for producing lipase,[3] have been proposed. Moreover, it has been studied its potential for environmental decontamination of heavy metals,[39] and for the biological pre-treatment of lignocellulose for bioenergy production.[20,45]

In Spain, pecan cultivation has been successfully expanded over the last two decades, particularly in Valle del Guadalhorce (Malaga). In this work, we tested the capability of *G. lucidum* to use lignocellulosic wastes of pecan.

Materials and methods

G. lucidum *strain isolation*

The fruiting body of a *G. lucidum* (Curtis) P. Karst specimen was collected from an evergreen oak (*Quercus ilex* subsp. *ballota*) forest in Robledo de Chavela (Madrid, Spain). The fruiting body was cultured in potato dextrose agar (PDA), at 25 °C in a culture chamber according to Postemsky et al.[31] The wild strain was first morphologically and anatomically characterized, as described in Nithya et al.[25] Secondly, the molecular analysis of the fruiting body by rDNA-ITS was obtained according to Zheng et al.[46] The fungal DNA was extracted and amplified by polymerase chain reaction (PCR) of the ITS region. The obtained nucleotide sequence was compared with those of GenBank, using the NCBI BLAST program. DNA sequences were aligned by MAFFT 7 using the default settings and manually optimized with BioEdit version 7.2.3. A phylogenetic analysis was carried out in PAUP version 4.0b 10. The specimen was identified through the sequence as *G. lucidum* (accession number: KT805317) (Appendix A). The cultures of *G. lucidum* were kept at 4 °C in the dark.[20]

The fungal radial growth (mm) in pure culture ($n = 10$) was registered according to Imtiaj et al.[15] to compare with commercial strains. Pure samples of *G. lucidum* cultures were kept on PDA in Petri dishes.

Spawn production

The spawn was produced with a mixture of grains of *Triticum durum* (59.1%, w/w), distilled water (40%, w/w), $CaCO_3$ (0.1%, w/w) to balance the pH, and $CaSO_4$ (0.8%, w/w) for the texture, according to Curvetto et al.[6] Two mycelial plugs of *G. lucidum* were inoculated. The spawn was produced after 10 days (0.25 l Erlenmeyer flasks at 25 °C in the dark).

Pecan waste preparation

The wastes of *C. illinoinensis* were obtained from an organic certified pecan plantation in Valle del Guadalhorce (Malaga, Spain). The pecan wastes were separated into three categories: (i) shells (PS), (ii) pericarp (PP) and (iii) branches (PB). First, the lignocellulosic wastes were dried at 35 °C for 3 days. Once dried, PP and PS were pressed, and PB was chopped into wood-chips. Then PS, PP and PB were sieved to obtain 2.5 and 5 mm particles. Pecan wastes were prepared according to Royse and Sanchez-Vazquez[32] and Lakshmi.[36] Finally, the pecan wastes PS, PP and PB were washed and hydrated with distilled water for 24 h. The relative humidity was adjusted between 60 and 70%.[29] In this study, the wastes were PS 100% (PS100), PP 100% (PP100), PB 100% (PB100), and the paired formulations PS50 + PP50, PB50 + PS50 and PB50 + PP50. All substrate formulations were supplemented with $CaCO_3$ 1% (w/w, dry matter) according to Yang et al.,[43] Erkel[8,9] and Manavalan et al.[20]

Experimental design

The experiment was carried out in glass tubes 20 cm long and 1.6 cm in diameter.[31] Tubes were filled with the different substrates to a height of 13 cm from the bottom (Fig. 1a). Nine replicates were prepared for each of the six substrates (eighteen tubes × 3) and sterilized in an autoclave for 45 min at 121 °C.[18,43] The glass tubes were inoculated at the top of the substrate with spawn at 5% (w/w) and were sealed with Parafilm M®. Colonization of the

Fig. 1. Lignocellulosic wastes of pecan studied for cultivating *Ganoderma lucidum*. (a) The six formulations include shells (PS100), pericarp (PP100), wood-chips of branches (PB100), and paired formulations of shells and pericarp (PS50+PP50), shells and wood-chips of branches (PP50+PS50) and pericarp and wood-chips of branches (PB50+PP50). (b) Fructification on the six formulations.

glass tubes with the different substrates took place in a chamber at 25 ± 1 °C. Mycelial growth rate (mm day^{-1}) was measured by marking the advancing fronts at intervals on the tubes, using the same method as for recording fungal radial growth on Petri dishes. To induce the formation of sporophore primordia, the tubes containing the mycelium were subjected to a cold shock for 2 days at 4 ± 1 °C.[29,44] Then they were placed in the growth chamber at 25 °C, with 85% relative humidity, and 10-h/day of light (fluorescent lamps; 500–1000 lux). A gap of 50 mm was left between the substrate and the Parafilm M® until the period of fruit body formation to promote gas exchange and the proper antler development. When the primordia appeared, the upper film was perforated and, once developed, the film was completely withdrawn.

Data analysis

The growth of *G. lucidum* was measured by estimating the biological efficiency, the production, and the dry biological efficiency. Biological efficiency[32] (BE) is defined as the weight of fresh mushrooms × 100/weight of dry substrate. Production[6] (P) is defined as the weight of fresh mushrooms × 100/weight of wet substrate. Dry biological efficiency[36] (DBE) is defined as the weight of dry mushrooms × 100/weight of dry substrate.

First, the assumptions of independence, normality and homoscedasticity were tested for the studied variables (mycelial growth rate, BE, P and DBE). Secondly, given that the data structure comply with normality criteria, ANOVA was used to evaluate the mycelial growth rate, BE, P and DBE, which were represented in terms of mean ± standard error. Analysis of variance (MIXED and REML) to test the differences in colonization and production between the substrates was carried out with Statistical Analysis System (SAS) software, version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Significant differences were determined by paired *Student-t* test (LSD) with $\alpha = 0.05$.

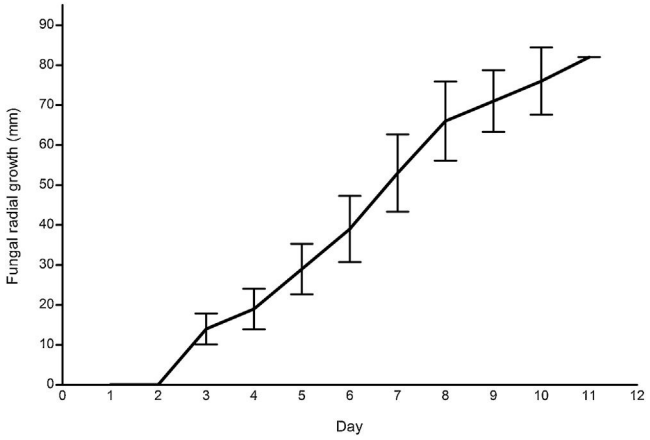

Fig. 2. Mycelium radial growth of the wild strain of *Ganoderma lucidum* ($n = 10$).

Results

G. lucidum *strain growth*

Fig. 2 illustrates the mycelial radial growth of *G. lucidum* ($n = 10$). The growth rate, 8.83 mm day^{-1}, was comparable to other wild strains, e.g., 8.70 mm day^{-1} from South Korea,[17] and

7.5–6 mm day^{-1} from India,[25] and to commercial strains.[11] Similarly, the mycelium grew consistently on wheat grains.

Mycelial linear growth on pecan substrate

All formulations were found to be suitable as solid substrate for *G. lucidum* when using 5% inoculum (Table 2). The mycelial growth was significantly higher in the PB50 + PS50 substrate, 5.54 ± 0.63 mm day^{-1} in a total of 23.67 ± 2.89 days to fill the tube. The lowest growth was recorded for the PB50 + PP50 substrate, 3.28 ± 1.20 mm day^{-1} over a period of 43 ± 13.75 days. The formulations PS100, PP100, PB100 and PS50 + PP50 had similar mycelial linear growth (Table 2).

G. lucidum *yield*

The six formulations were able to produce at least two flushes over the period of the experiment (Fig. 1a and b). Table 2 shows the performance in mushroom yield of *G. lucidum*. The BE ranged from 4.36% for substrate PP100 to 55.42% for substrate PB100; P ranged from 1.43% in the substrate PP100 to 22.97% in the substrate PB100, and DBE ranged from 0.66% in the substrate PP100 to 11.08% in the substrate PB100.

Discussion

G. lucidum has been successfully produced on a wide variety of waste materials, including corn stover residues,[33] sawdust with rice straw,[40] sunflower seed shells,[11] tea residues,[26] bagasse from sugar cane,[20] soy residues,[14] fish waste[36] and different types of sawdust[2,8,18] (poplar, beech, hornbeam, oak) (Table 1). Similarly, studies on lignocellulosic degradation ability have been conducted. Zhang et al.[45] studied the biological pre-treatment of bamboo culms (*Phyllostachys pubescence*) with *G. lucidum* and 33 other WRF. After a 4-week period of cultivation, *G. lucidum* caused component loss of 12.10% (w/w) in weight: 10.56% (w/w) in lignin, 12.83% (w/w) in cellulose, and 15.16% (w/w) in hemicellulose. The selective delignification of *G. lucidum* was also observed in poplar wood.[19] Table 1 reviews the available studies on solid substrates for cultivating *G. lucidum*, using both commercial and wild strains. Comparison with the pecan wastes associated with BE and DBE is illustrated in Fig. 3a and b.

The *G. lucidum* strain KT805317 used for the first time in this study, is adapted to Mediterranean climate conditions, contrary to the strains reported so far (Table 1). The results were particularly significant regarding the proportion of the native inoculum used in the degradation of residues. The mycelial growth rate of *G. lucidum* in pecan waste was analogous to that on other solid substrates, such as agro-industrial rice residues on substrate for-

Table 1
Solid substrates with the highest performance in the cultivation of the medicinal *Ganoderma lucidum* mushroom.

Substrate formulation			BE (%)	Region	Reference
Highest performance (%)	Substrate	Supplement			
SB100	Sugarcane bagasse (SB)	$CaCO_3$	80 ± 15	Tamil Nadu, India[a]	20
MS80+WB20	Sawdust of sheesham, mango (MS) and poplar, wheat brans (WB), rice bran, corn flour	Gypsum and $CaCO_3$	58.57	Himachal Pradesh, India[a]	21
HS80+TW20	Tea manufacturing waste (TW), Sawdust of hornbeam (HS), wheat bran	Sucrose and $CaCO_3$	34.9	Denizli, Turkey[b]	26
SDM22.5+PA67.5+RB10	Sawdust mixture (SDM), paddy straw (PA), rice bran (RB)	None	29.9	Rajasthan, India[a]	40
PSD58+BSD29+RG13	Poplar sawdust (PSD), beech sawdust (BSD), rye grain (RG)	$NH_4H_2PO_4$ and $CaCO_3$	25.5	Not indicated[b]	27
AS90+GF10	Sawdust of *Alnus nepalensis* (AS), *Shorea robusta, Dalbergia sisoo*, rice bran, wheat bran, corn flour, gram flour (GF)	Gypsum and $CaCO_3$	22.62	Imadole, Nepal[b]	12
BP100	Billets of poplar (BP)	Malt extract	22	Uttarakhand, India[a]	34
PSD80+WB20a	Sawdust of poplar, oak, beech, brans of wheat, rice, corn	Gypsum and $CaCO_3$	20.85	Marmara, Turkey[a]	8
PSD80+WB20b	Poplar sawdust, wheat bran	Molasses and corn gluten meal	20.37	Marmara, Turkey[a]	9
PSD94.5+ME5.5	Sawdust of poplar, beech, hornbeam, wheat bran, malt extract (ME)	$CaCO_3$, Gypsum and KH_2PO_4	18.68	Mashhad, Irán[b]	2
RS67+RH25+RB8	Rice straw (RS), rice husk (RH), rice bran	Olive oil	13.8	Guelph, Ontario, Canada[b]	31
SB100	Fishery waste, coir pith, wood-chips, sugarcane bagasse	None	12.95	Tamil Nadu, India[a]	36
SSH85+WB5	Sunflower seed hull (SSH), wheat bran, malt	Gypsum and $CaCO_3$	10	Olympia, WA, USA[b]	11
			DBE%		
BS80+WB20	Oat straw, bean straw (BS), *Brachiaria* grass straw, *Tifton* grass straw, *Eucalyptus* sawdust and wheat bran	$CaCO_3$	6.7	Botucatu, Brazil[b]	22
ASD50+SG30+WB20	Sawdust of stalk of *Acacia confusa* (ASD), Stillage grain (SG), wheat bran	$NH_4H_2PO_4$ and $CaCO_3$	5.36	Hsinchu, Taiwan[b]	43
FWC15+RB17+OS68	Food waste compost (FWC), rice bran and oak sawdust (OS)	NaCl and Ca	3.4 ± 0.2	Chuncheon, South Korea[b]	18

[a] Wild fungal strain.
[b] Commercial fungal strain.

Table 2
Ganoderma lucidum growth on the six formulations of pecan wastes. Mycelium linear growth rate, biological efficiency (BE), production, and dry biological efficiency (DBE).

Substrate	Mycelium linear growth rate (mm/day)	BE (%)	P (%)	DBE (%)
PS100	4.05 ± 1.39^{ab}	17.57 ± 0.56^{d}	8.79 ± 0.28^{c}	3.72 ± 0.43^{d}
PP100	4.00 ± 1.83^{ab}	4.36 ± 3.28^{e}	1.43 ± 1.08^{e}	0.66 ± 0.72^{e}
PB100	4.62 ± 1.45^{ab}	55.42 ± 14.72^{a}	22.97 ± 6.10^{a}	11.08 ± 2.95^{a}
PS50+PP50	5.00 ± 1.03^{ab}	11.85 ± 5.28^{de}	4.92 ± 2.19^{d}	2.37 ± 1.06^{d}
PB50+PS50	5.54 ± 0.63^{a}	25.37 ± 2.91^{c}	11.87 ± 1.36^{b}	5.07 ± 0.59^{c}
PB50+PP50	3.28 ± 1.20^{b}	31.66 ± 1.71^{b}	11.43 ± 0.62^{b}	6.33 ± 0.35^{b}

The same letters are not significantly different by the paired student-*t* test ($\alpha = 0.05$).

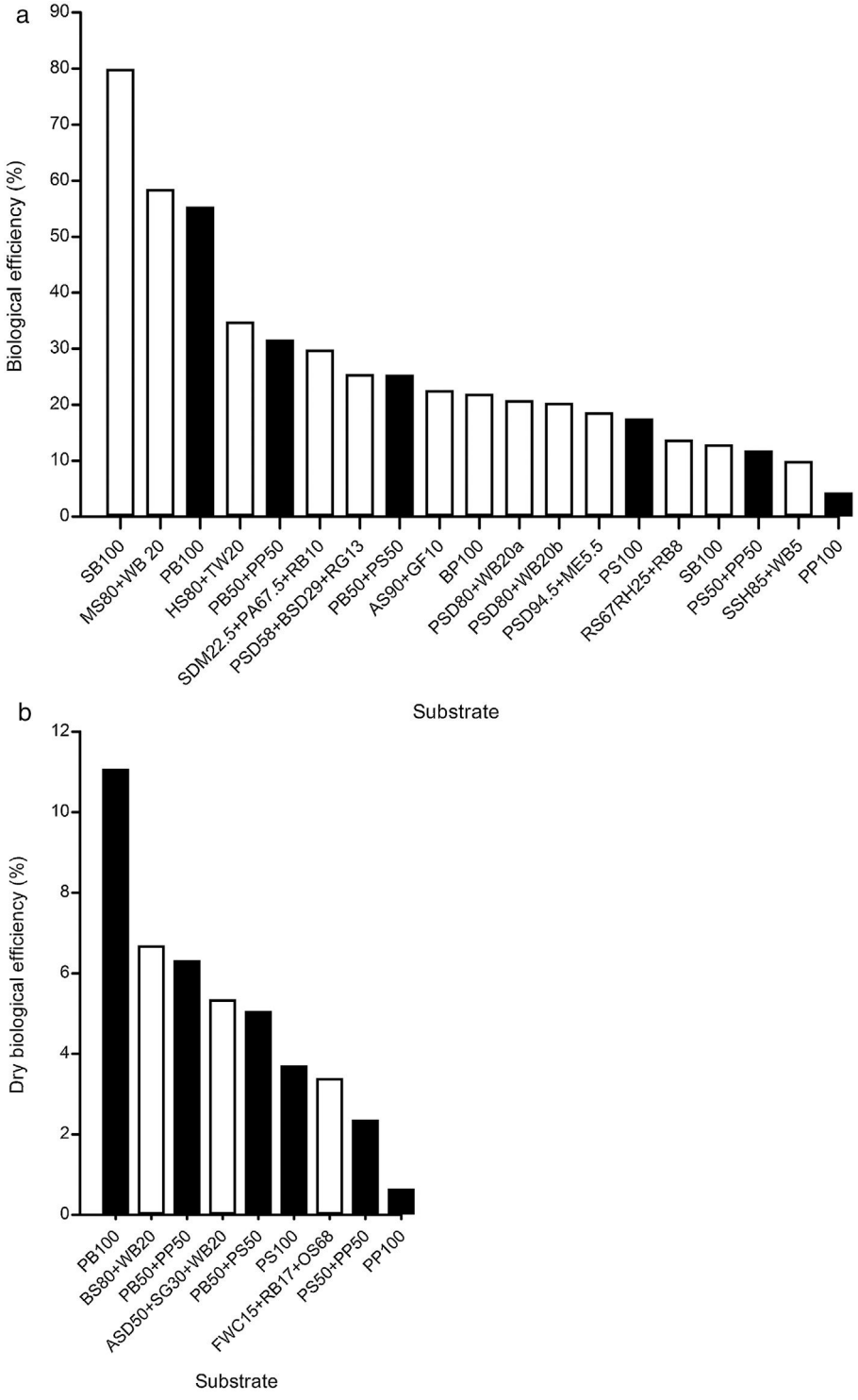

Fig. 3. *Ganoderma lucidum* mushroom cultivation in solid substrates. (a) Biological efficiency, and (b) Dry biological efficiency. Formulations of pecan wastes (black bars) are compared with other solid substrates (white bars); abbreviations of the solid substrates are given in Table 1.

Table 3
Orthogonal contrast of yields in relation to the substrates with and without wood-chips of pecan branches (PB).

Index	With PB	Without PB	Difference
BE	37.4844	11.2611	26.2233***
P	15.42	5.0456	10.3744***
DBE	7.4956	2.2478	5.2478***

BE: biological efficiency, P: production, DBE: dry biological efficiency.
*** p-Value < 0.01.

mulations using 10% of inoculum[20,43] and 8% of inoculum.[31] The wood-chips formulations with shells or pericarp improved the *G. lucidum* yield. The biological efficiency increased from 4.36% in PP100 formulation to 31.67% in PB50 + PP50, and from 8.79% in PS100 formulation to 11.87% in PB50 + PS50 (Table 2). The orthogonal contrast (Table 3) corroborates the importance of formulations in the mushroom yield, with significantly higher values of BE, P and DBE in the substrates with pecan wood-chips (PB100). Ultimately, substrate PB100 was certainly the most efficient for *G. lucidum* yield. The higher similarity of the substrate formulations with the natural environment in which the *G. lucidum* grows could be possibly the key for success to obtain high BE values.

No significant correlation was observed between the rate of mycelial growth and mushroom yield within the same test formulation, ρ (mycelial growth rates, P) = 0.1430, p-value = 0.5713 (ns) and ρ (mycelial growth rates, BE) = 0.0927, p-value = 0.7144 (ns). It is important to know how long *G. lucidum* takes to colonize each type of substrate. This is an industrial setting of great interest, but should not be taken as a measure of performance. This result indicates that a substrate should not be discarded for a long colonization time.

This study shows the novelty of using pecan wood-chips instead of sawdust to produce *G. lucidum*. Royse and Sanchez-Vazquez[32] reported the influence of wood-chip particle size on the cultivation of the shiitake (*Lentinula edodes*); yields from substrates prepared with wood-chip particles of <0.85 mm were compared with yields from substrates prepared with wood-chip particles of 2.8 ± 4.0 mm. Further research is required to confirm the influence of particle size of pecan wastes on G. *lucidum* cultivation and yield.

The pecan wood chip formulation (PB100), with 55.42% BE, emerges as one of the best solid substrates for cultivating *G. lucidum* (Fig. 3a), only surpassed by sugarcane bagasse (SB100), with 80 ± 15% BE,[20] and sawdust of mango and wheat brans (MS80 + WB20), with 58.57% BE.[21] Both the formulations PB50 + PP50, with 31.7% BE, and PB50 + PS50, with 25.4% BE, are listed in the top ten agro-industrial residues to obtain *G. lucidum* mushrooms. The only additive used in our study was the pH regulator (1% of $CaCO_3$). Comparable results in the same conditions were those of pecan shells (PS100), with 17.6% BE, and the mixture of poplar sawdust and wheat bran without additives (PSD80 + WB20), with 17.2% BE.[9]

The DBE index is particularly relevant for *G. lucidum* production because this mushroom is being sold as dry matter in all its forms (powder, capsules, whole and chopped). The best value was obtained for PB100 (11%) relative to other reported values (Fig. 3b).

Conclusions

This study describes an attempt to cultivate the medicinal mushroom *G. lucidum* by using agro-industrial residues of *C. illinoinensis*. The results show that all six formulations of the substrates tested were suitable for the fungus growth and mushroom yield. The mushroom yield was increased with formulations including shells or pericarp with wood-chips. The best result (55.4% BE) was obtained with PB100. The cultivation of pecan is being expanded in both the northern and southern hemispheres[41] due to market demand and the ability of the tree to adapt to a range of climate environments.[4] The economic aspect of pecan production on a global scale could benefit considerably from the usage of the lignocellulosic wastes to produce high value products in the form of mushrooms with medicinal and biotechnological applications.

Conflict of interests

All authors declare that they have no conflict of interest.

Acknowledgements

We are grateful for the support and materials provided by the producers of pecan nuts from the Asociación Valle del Guadalhorce, in the province of Málaga, Spain. We also thank Álvaro Rodrigo for providing the *G. lucidum* specimen. This work was possible due to the funding provided by the Erasmus Mundus Program under a EuroTango Project for a PhD program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.riam.2017.09.005.

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