A survey and molecular identification of *Aspergillus versicolor* causing brown rot on imported spruce (*Picea canadensis*) wood in Karbala province, Iraq and control it using Copper Boron Chromate

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**Abstract.** This study aimed to survey, molecular diagnosis and assessment effect of *Aspergillus versicolor* on the imported spruce wood *Picea Canadensis* Miller. in Karbala province, Iraq, and control it using Chromate Copper boron Chloride (CCB). Result of survey showed that all imported woods were showing different level of brown rot symptoms in Kerbala province, Iraq. The fungus associated with this symptom was isolated and identified based on its morphological and molecular characterizations as *A. versicolor*. The deterioration efficiency of the isolated fungus was assessed on wood *P. canadensis* which showed subsequently a high effectiveness in decomposing wood tested causing a significant reduction in quality. This identification is the first record of this fungus affecting the imported wood *P. canadensis* Miller. in Kerbala province, Iraq. Furthermore, it was noticed that the growth of *A. versicolor* was completely inhibited by CCB at concentration (3000 mg/L). Moreover, this preservative material provided a complete protection to woods examined for the two periods of incubation (three and six month) with fungal inoculation in addition to occurrence of a significant increase in their thickness and dry weight.

1. Introduction

Wood is a very valuable supply in this world and is one of the largest sources of energy available on earth. It consists of various organic compounds such as the cellulose, hemicellulose and lignin. The percentage of cellulose in wood is ranged between 40-50% of dry weight. It has been employed as a basic material in the manufacture of paper, glucose, ethyl alcohol, cellulose, plywood, gypsum wood, granular, coal, carpentry and other more materials [1].

Fungi under certain conditions are one of the most important and dangerous living organisms that affect wood. They destroy the internal structure of wood by affecting its main component such as cellulose, hemicellulose and lignin [2]. Worldwide, these fungi cause a significant economic loss.
For example, in the United States the loss of untreated wood was estimated as 50 million dollar per year due to these fungi impact [3; 4]. There are a variety of wood-decay fungi that cause different wood rot such as the brown rot. The brown-rot fungi break down hemicellulose and cellulose. Worth to mention that the cellulose is damaged by hydrogen peroxide (H$_2$O$_2$) that is produced during the break-down of hemicellulose. This is because of H$_2$O$_2$ is a small molecule that can diffuse rapidly through wood leading to a decay. As a result of this type of decay, the wood shrinks, shows a brown discoloration, and cracks into roughly cubical pieces [5].

The conservation treatments of woods include utilizing the chemical materials that may provide protection to the wood through inhibition activities of decay fungi and increase the strength of wood [6]. The CCB is one of these chemical treatments that has been widely employed in wood preservation [1]. For example, the CCB provided a significant protection to wood treated from brown rotting. However, this protection differentiated due to presence of different fungi, especially under wetting conditions. These conditions caused various destructive distortions and disintegrations of wood [7].

Like other members of its species Aspergillus versicolor is an opportunistic pathogen and is considered to be an important causative agent of wood rot [8; 9]. According to previous studies [10,11] the A. versicolor was among the most common of indoor molds, often reported in dust and in water-damaged building materials, such as wallboards, insulation, textiles, ceiling tiles, and manufactured wood, and is a highly resilient fungus, explaining its wide global distribution in a variety of environmental conditions. Although, it grows optimally between 22 and 26 °C, it can grow at temperature ranged 4–40 °C. The fungus can also tolerate a wide pH ranges, and is particularly resistant to alkaline conditions [12]. Additionally, A. versicolor produces the xylanase, which is an enzyme breaking down the cellulose and hemicellulose in plant cell walls and degrades xylan in waste products from hardwood manufacturing and agricultural activities [13; 14].

In Iraq particularly in Kerbala province, there have been no studies accomplished regarding to the nature of wood conservation, especially with spread of deterioration caused by various fungi on imported spruce wood. Thus, the objects of this study were survey and molecular identification of the causal agent associated with brown rot of the imported spruce wood and evaluation the effectiveness of the CCB in preservation of this wood from brown rot.

2. Materials and Methods
2.1. Survey of degraded spruce woods
A survey of imported spruce wood was carried out in five main wood shops for manufacturing and selling of woods, as well as a number of stores in different regions of the holy city of Kerbala, Iraq in May 2015. It was shown through the apparent symptoms of brown rot through working of sections in the wood sampled randomly. The infected wood was classified into five categories based on evidence of the severity of deterioration described previously [15] that was calculated [16] using the following equation:

\[
\text{Disease severity} = \frac{\text{N. of woods X category (0)} + \ldots + \text{N. of woods X category (4)}}{\text{Total number of wood surveyed} \times \text{top category index (4)}} \times 100
\]

The percentage of degraded wood was calculated as following:
Number of infected wood

\[
\text{Disease incidence} = \frac{\text{Number of infected wood}}{\text{Total number of wood surveyed}} \times 100
\]

2.2. Isolation and morphological identification of the fungus associated with degraded spruce wood

Samples of degraded spruce wood were brought to the laboratory to isolate the fungi causing the deterioration. The symptomatic parts were washed with tap running water for an hour. Sections (0.5 cm long) from adjacent areas of deterioration were then cut with a sterile scalpel. These sections were sterilized with 1% sodium hypochlorite for three minutes, washed with distilled water and dried using sterile filter paper (Whatman No.10). The sterilized pieces were placed in each sterile petri dish (9 cm in diameter) containing sterile Malt Extract Agar (MA) medium amended with Rose Bengal 30 mg/l [17] in addition to the antibiotic chloramphenicol (250 mg/l). Each dish contained 5 pieces of wood. Subsequently, all petri dishes were incubated in darkness at 25 ± 2 °C for 3 days. Colonies were purified and morphologically initially identified as Aspergillus versicolor based on previous descriptions [8; 9; 10].

2.3. Evaluation the isolated fungus in causing deterioration on wood spruce

Wooden boards were selected randomly from a spruce-free injury obtained from a commercial shop in Kerbala city. This free wood boards from injury were cut in pieces with standard dimensions (3 x 1 x 5.0 cm long) [18]. These wood pieces were then autoclaved and dried in an oven at 105 °C for 48 hours (until stability weight). After that the wooden pieces were inoculated with the fungus isolated by putting them in bottles containing the fungal inoculum growing on infected pieces of wood. All bottles were subsequently incubated at 25 ± 2 °C for 9 weeks [19]. Afterward, the inoculated wooden pieces were washed, dried and weighed [20]. The fungus was re-isolated and the proportion of lost weight of the pieces as evidence of the extent of deterioration was calculated. Additionally, rectangles wooden pieces with dimensions of 6 x 2 x 2 cm long were autoclaved and dried as previously. This test was repeated longer period 3 and 6 month [18].

2.4. Molecular identification of the fungus associated with degraded spruce wood

The genomic DNA of a pure fungal colony was extracted by the DNeasy Plant Mini Kit (QIAGEN N.V., Hilden, Germany). The two primers (ITS1 and ITS4) were employed to amplify the internal transcribed spacers of ribosomal DNA (ITS-rDNA) [21]. The Ready-To-Go PCR Beads kit were exploited in a total volume of 25μl solution containing the basic ingredients provided by the company as beads and 1μl of each primer (5 pmol) in addition to 2 μl (50-100 ng) of template DNA (GE Healthcare, Illinois, US). The PCR products were sequenced at Macrogen, Inc. (Seoul, South Korea). The sequences collected were compared with others ITS-rDNA sequences of fungi deposited at GenBank sequence database of NCBI applying the BLAST (Basic Local Alignment Search Tool) program. Then, the phylogenetic analyses of the sequences data was executed using MEGA (Molecular Evolutionary Genetics Analysis) version 6.0. [22] The generated sequence was submitted to GenBank database and assigned with special an accession number.

2.5. In vitro assessment effect of Copper Boron Chromate (CCB) preservative on A. versicolor isolated

The CCB was added to the MA medium and mixed thoroughly before solidifying and then poured in 9 cm sterile Petri dishes. As well as the antibiotic chloramine phenolic (250 mg/l) was added into MA medium and poured into sterilized Petri dishes to be used as control. After that, 4 mm in
diameter disks collected from the edge of the pure 5-days-old fungal culture of were placed in the centre of each Petri dish prepared. These inoculated Petri dishes were later incubated at 25 ± 2 °C in darkness. The measurement of two perpendicular diameters of growing fungal colony was calculated and the percentage of inhibition of the mycelia growth was calculated as following:

\[
\text{Inhibition} \% = \frac{\text{Average growth of control colonies} - \text{Average growth of treatment colonies}}{\text{Average growth of control colonies}} \times 100
\]

Additionally, the relative amounts of changing in thickness and weight after immersion in water for a period (48 hour) were measured from three points. The samples were bathed in a basin filled with water (5 cm deep) for a period (48 hours). The samples were then taken out and the weight and the thickness were measured for all samples. The percentage of increase in thickness and weight was calculated using the following equation:

\[
W_{48} \% = \left(\frac{w_{48} - w_1}{w_1}\right) \times 100 \quad \text{(1)}
\]

\[W_{48} \% = \text{Percentage change in weight after water immersion 48 hours.}
\]

\[w_{48} = \text{Sample weight after 48 hours of immersion (g).}
\]

\[w_1 = \text{Sample weight before 48 hours of immersion (g).}
\]

\[
T_{48} \% = \left(\frac{T_{48} - T_1}{T_1}\right) \times 100 \quad \text{(2)}
\]

\[T_{48} \% = \text{Percentage change in thickness after water immersion 48 hours.}
\]

\[T_{48} = \text{Sample thickness after 48 hours of immersion (g).}
\]

\[T_1 = \text{Sample thickness before 48 hours of immersion (g).}
\]

2.6. In vivo assessment effect of CCB preservative on A. versicolor isolated

A %5 of the conservation material of solution CCB 34 % 5H 2O × CuSO4 and %37 K 2 Cr 2 O7, and %28 H3BO3 was prepared [23]. The wood pieces were submerged in the CCB solution for five days. They were then removed and dried in an oven at 60 °C for 24 hours. The face representing the largest surface area of the pieces was placed directly on the developing colonies of A. versicolor and incubated at 25±2 °C and for periods (0,3,6 month) [18]. During the incubation, the effect of A. versicolor and incubation periods in the loss of wood weight was evaluated by collecting the wood pieces after each period and washing and drying them then weighed by a sensitive balance. Each treatment included three replicates, and the percentage of weight loss was calculated as the following equation:

\[
\% \text{weight loss} = \left(\frac{\text{Weight of the wood pieces before inoculation} - \text{weight it after inoculation}}{\text{Weight them after inoculation}}\right) \times 100
\]
All experiments were carried out using a completely randomized design (CRD); the averages were then compared using a test least significant difference (L.S.D) at a probability level of 0.05.

3. Results and Discussion
3.1. Survey of degraded spruce woods
The results of the survey (Table 1) showed that the percentage of brown rot incidence ranged between 91 - 100% with severity rated between 0.91 to 0.96 in Kerbala province. It is clear from the results of the intensification deterioration of spruce wood in Kerbala province which is due to expose to the fungal agent for a long time since its existence in the stands or forest and during storage as well as the timber manufactured the effects of brown rot appear clearly (Figure 1). It is expected that the infection was vascular while no symptoms of brown rot on the surface of the wood was showed [24].

Table 1. The incidence and severity of brown rot spruce wood deteriorate in the holy province of Karbala

| Regions       | Brown rot incidence (%) | Brown rot severity |
|---------------|-------------------------|--------------------|
| Al-gerah      | 91                      | 0.96               |
| Bab Touarej   | 100                     | 0.91               |
| Al-Husseinia  | 100                     | 0.94               |
| Al-Abasia     | 95                      | 0.93               |

Figure 1. Areas of deteriorate brown rot symptoms in stored spruce wood.

3.2. Isolation and morphological identification of the fungus associated with degraded spruce wood
Colonies vary greatly in colour, growth rate, and surface characteristics depending upon growth conditions. Colonies were typically white at the start of development, and changed to yellow, orange, and green, often with pink or flesh hues intermixed, at maturity. Conidia were very in their size and were single or chained. Their size and shape were similar to those of *A. versicolor* [25].
3.3. Evaluation the isolated fungus in causing deterioration on wood spruce
This test revealed that the fungus was able clearly to cause the deterioration on wood tested. Moreover, it caused loss (4.20%) of wood weight during period of 3 months (Figure 3). The loss of the dry weight of spruce may be due to the exploitation of its main chemical components as nutrients to the *A. versicolor* in their growth and reproduction [1].

**Figure 3.** Testability of deterioration to the isolated fungus After 3 months of incubation period

3.4. Molecular identification of the fungus associated with degraded spruce wood
The molecular identification result confirmed the initial morphological identification to the causal agent of brown rot that was the fungus *A. versicolor*. Phylogenetic analysis demonstrated the close relationship between *A. versicolor* isolated in this study and others worldwide strains (Figure 4).
3.5. In vitro assessment effect of Copper Boron Chromate (CCB) preservative on A. versicolor isolated

The results shows in figure (5) that the preservative had a significant effect in inhibiting the growth of maysliom from the average concentration effect, the efficiency of the preservative is increased by increasing the concentration gradually and better by inhibiting the fungus A. versicolor at a concentration of 3000 mg/l at a rate of 100%.

Many preservatives have been used as inorganic substances to support the active ingredient in insecticides for the control of woody fungi as well as high toxicity and role in the inhibition of fungi.
as these chemicals are changing the nature of protein and enzymes fungal association with the groups (SH) In the fungus cells and thus by many vital actions, including breathing [26] This also applies to the rest of the studied substances, when used all of the effectiveness of high inhibition of fungal enzymes, although varied in efficiency against fungus[1].

3.6. In vivo assessment effect of CCB preservative on A. versicolor isolated
Table (2) shows that there was a significant increase in the weight of A.versicolor wood, recording a rate of 21.84 CCB had the ability to maintain wood from the weight increase of wood Infected with A.versicolor, recording a 17.11 rate of increase in weight for control. the increase in weight increased significantly after six months,And for the effect of overlap we note that the fungus worked to increase the percentage of weight of wood and significantly compared to the treatment of the comparison rate of 20.53 and 23.14%, respectively Thus, the CCB was able to reduce the injury and gave good values in preserving wood treated compared to untreated wood.

Table 2. Effect of the CCB preservative in the protection of spruce models from weight increase after 48 hours of immersion in water

| Treatments       | 3     | 6     | Effect of the average treatments |
|------------------|-------|-------|---------------------------------|
| Comparison (0)   | 17.95 | 17.95 | 0.18                            |
| A. versicolor    | 20.53 | 23.14 | 21.84                           |
| CCB              | 17.11 | 17.23 | 0.22                            |
| Effect of the average periods | 18.53 | 19.44 |                                |

The least significant difference at the level of probability 0.05, Periods= 0.70, Treatments=0.86, Interference=1.22

Table (3) and Figure (6) show a significant increase in the percentage of fish bloating in the fungus, where the rate of swelling of thickness for spruce was 16.77% after 48 hours of water immersion Compared with control treatment and treated wood with CCB, while the treated wood did not differ significantly from the comparison.Periods did not have a significant effect on this characteristic. We found that CCB was efficient in wood conservation for the increase in thickness, at 9.12% and 9.23%, respectively (Which did not differ significantly from the comparison) compared to the treated fungus only registered rate of 15.59 and 17.95% respectively and for the same periods.

Table 3. Effect of the CCB preservative in the protection of spruce models from thickness
Through the results of the sample studied and shown in the two tables above, the fungus caused a decrease in the percentages of the dry weight of spruce wood as a result of the fungi feed on carbohydrates, their growth and reproduction. This corresponds to what he said [6] wood hasn't treated with preservatives, be prone to degradation to feed the fungi on carbohydrates. That the injury of wood as a result of the fungal attack will destroy cellulose by enzymatic factors and hemicellulose by enzymatic and non-enzymatic factors such as free radicals such as Fe ++ compound Iron ions holder Which Turns in reverse interaction to oxalic acid or to Fonton H2O² where a group is produced Hydroxyl radical (HO) containing free radical oxygen (O²) and the product of hydrogen peroxide reaction with iron salt Fe ++ by interaction Fonton [24] The primary wall openings are very small and cannot be penetrated by innate enzymes, especially during the initial stages of degradation. They act together as non-enzymatic agents in the breakdown and oxidation of hemicellulose, as well as the breakdown of cellulose and thus feeding them causing loss of the dry weight of wood [27]. There found the role of fungi in feeding on carbohydrates and thus increasing the permeability and diffusion of liquids, leading to high absorb in decaying woods, while remaining prone to degradation and decomposition continuously when other growth factors are available and woody destroying colonies increase, causing a decrease in strength before the reduction in Weight will therefore affect its physical properties. CCB preservative is important in reducing the degradation of treated wood compared to unsaved timber and its obvious role in killing the fungus or reducing its activity leading to reduce the degradation in the wood and reduce the dry weight loss resulting from the effectiveness of the fungus. Its role in inhibition of enzyme action and effect on fungal cell [1]. The role of preservatives, such as CCB, is shown as a water-based preservative that inhibits the growth of brown rotting fungi of wood with good and acceptable proportions Each substance has its own role of toxicity of copper sulfate, which when mixed with boric acid will give a higher inhibition as well as the presence of potassium dichromate, which has an effective role in the stabilization of copper with wood when the process of conservation [28].

| The incubation period month | Treatments | 3 | 6 | Effect of the average treatments |
|----------------------------|------------|---|---|-------------------------------|
| Comparison (0)             |            | 9.42 | 9.42 | 9.42                        |
| A. versicolor              |            | 15.59 | 17.95 | 16.77                        |
| CCB                       |            | 9.12 | 9.23 | 9.17                        |
| Effect of the average periods |          | 11.37 | 12.20 |

The least significant difference at the level, of probability 0.05, Periods= 0.89, Treatments=1.09, Interference=1.55
Figure 6. Test samples after six months of incubation: 1- control, 2- CCB and fungus, 3- fungus only

Treatment with preservative materials has proven to prolong the period of use of wood by 5 to 10 years [29] in addition to improving their different properties, it is preferable to treat wood preservatives after cutting them directly from the trees and before storing them or manufacturing them to be more economically and industrially efficient.

4. Conclusions
In the present study, it was achieved a first molecular confirmation of A. verseicolor as causal agent of brown rot on imported spruce wood in Kerbala province, Iraq. It was also realized a significant effectiveness of CCB against growth of the brown rot causative agent, A. verseicolor.

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