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Detection and Identification of Fungal Diversity Associated with Soybean (*Glycine max* (L.) Merrill) Seeds from the United States with Blotter Testing Method

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Abstract

Soybean is one of the most favorite commodities of Indonesian people. Soybeans, as a potential agricultural product, need to be increased in production because they are the main source of protein for most families in Indonesia. This study aims to detect and identify the diversity of fungi carried by soybean seeds (Glycine max (L.) Merril) from the United States using the Blotter Testing method on different substrate variations. The method used in this study is a qualitative descriptive method with an observational method. This study employed a non-factorial Randomized Block Design (RBD) with three substrates: Filter Paper (S1), Opaque Paper (S2), and Kitchen Tissue (S3). The study's results identified two types of fungi in soybean seeds from the United States through blotter testing, based on morphological characteristics: Rhizopus sp. and Aspergillus sp. Notably, Aspergillus sp. was found with the highest frequency. The application of the blotter method using filter paper gave the best results in identifying fungi based on the high frequency of fungal findings. The presence of both types of fungi indicates that soybeans are dangerous for consumption because they contain Aflatoxin compounds.

Keywords: Fungi, blotter method, soybeans

PENDAHULUAN

Soybeans (Glycine max (L.) Merril) are one of the most popular commodities in Indonesia because soybeans are the main source of vegetable protein for most people. The volume of soybean production in Indonesia continues to experience a deficit throughout the year due to the demand for soybeans, which is not comparable to the domestic soybean production capacity. Referring to the data published. Central Statistics Agency (BPS) 2023, Indonesia's soybean production volume only reached 555,000 tons, while the national soybean requirement reached 2.7 million tons. This makes the government rely on imported soybeans for domestic soybean needs, one of which is the United States. This is based on data from the Central Statistics Agency (BPS) for Soybean Import Data by Main Country of Origin in 2023 where the United States is the largest soybean importer to Indonesia with a volume of 1,949,365.2 metric tons or worth 1.26 billion US dollars, which shows the major role of the Superpower in fulfilling the global soybean supply chain, including Indonesia.

Based on the description above, the entry of imported soybeans into Indonesia poses a significant risk as a carrier of disease-causing pathogens that can affectplants in Indonesia. This can cause various negative impacts, such as material losses due to fungal infections in soybean cargo during shipping, resulting in the failure of shipments to enter Indonesia. According toHayati et al. (2022), soybeans originating from import trade are one way for pathogens to spread at their destination and risk causing disease and material losses, which are caused by their spread through the soybean seeds themselves. One of the pathogens often found in imported soybeans is a fungus. Noerfitriyani and Hamzah (2018), define fungi as microorganisms that affect plant growth and can be endophytic or pathogenic. According to Yani et al.

(2020), fungi that do not have chlorophyll generally survive by utilizing residue or organic material from other microorganisms, so that they are heterotrophic. As a result, this disrupted the supply chain and caused soybean prices on the market to soar. This study aims to detect and identify the diversity of fungi carried by soybean seeds (Glycine max (L.) Merril) from the United States using the Blotter Testing method on different substrate variations.

RESEARCH METHODOLOGY

This type of research is a qualitative descriptive study with an observational method. This research was conducted at the Laboratory of Basic Sciences and Plant Protection, Faculty of Agriculture, Sultan Ageng Tirtayasa University, and the Insect Laboratory, Animal, Fish, and Plant Quarantine Center, Banten, Merak Service Unit, Grogol District, Cilegon City, Banten. This research was conducted from February to April 2024. The tools used in this study were Laminar Air Flow, measuring cups, petri dishes with a diameter of 9 cm x 9 cm, infrared sterilizer, stereo microscope, compound microscope (Leica and Compound), digital microscope (Hirox), plastic clips, stopwatch, cutter, gloves, digital scales, sprayer, stationery, books, and cellphone cameras. Meanwhile, the materials used in this study were soybean seeds from the United States taken from the BKHIT Banten laboratory, distilled water, 1% sodium hypochlorite (NAOCl), 70% alcohol, masks, tissues, filter paper with a pore diameter of 25 µm, opaque paper, and kitchen tissue. This study used a non-factorial Randomized Block Design (RBD) with three levels, namely substrate variations including Filter Paper (S1), Opaque Paper (S2), and Kitchen Tissue (S3). This study was conducted in two stages, namely data collection and the stage of fungal identification in the laboratory. The observation parameters in this study include the following:

a. Morphological Characteristics

Morphological characteristics are special descriptions to describe and identify a species based on morphological conditions. This is useful in the availability of descriptive terms to distinguish species from other species so that grouped species are obtained. According to(Sine & Soetarto, 2018),The morphological characteristics of fungi are as follows Table 1.

Table 1. Morphological Characteristics of Fungi

Character	Information
Spore color	Spores can be white, black, or green,
Mycelium color	Mycelium is generally white in color.
Sporangiophore texture	Texture can be seen either smoothly or roughly.
Columella shape	Shaped round or imperfectly round
Spore form	Spores can be round, oval, or imperfectly round.
Rhizoid	Rhizoids generally grow in the opposite direction to the sporangiophores.
Colony form	Shaped
Types of hyphae	Septate or axeptate
Identification	Types identified based on the above information

Description: Colonies are distinguished based on differences in colony color during direct observation and under a microscope.

b. Frequency of Fungi Findings

The frequency of fungal findings is the proportion of samples showing the presence of fungi compared to the total samples tested. The frequency of fungal findings refers to a parameter that indicates how much or how often fungi with specific characteristics are seen in a population in the entire test that has been conducted. According toLuturyali et al. (2019), The frequency value can describe environmental conditions during testing and can be calculated using the following formula:

Frequency of Findings =

$$\textbf{x100\%}\frac{\sum \textbf{Cendawan Teridentifikasi pada Benih}}{\sum \textbf{Benih Diinkubasi}}$$

c. Fungal Diversity Index

Fungal diversity index refers to the variation of types (species) of fungi found in the tested samples, as well as their distribution and abundance measured using diversity percentage to understand the proportion of fungi in soybean seeds incubated with various substrates. The diversity index can be measured by the Shannon-Wiener diversity index (Anjani et al., 2022):

$$\mathbf{H' = -\sum Pi \ ln \ (Pi)}$$

where:
H': Shannon-Wiener Diversity Index
Pi : ni/N
ni: Number of individuals of type i
N : Total number of individuals of all species

The value of the diversity index according to Shannon-Wiener ranges from 1-3, which can be categorized as follows(Anjani et al., 2022):

H' = < 1, including low-level diversity
H'= 1-3 Includes a moderate level of diversity
H' = > 3 including a high level of diversity

d. Species Evenness Index

The species evenness index aims to be an indicator to determine the balance of the distribution of individual species found in a community or vegetation.Baderan et al. (2021),states that the species evenness index is calculated using the Margalief index, namely:

$$\mathbf{E = \frac{H'}{In\ (S)}}$$

E: Species Evenness Index
H': Species Diversity Index
S: Number of Species

The categories for determining species wealth for the Margalef Wealth Index are as follows:

E = 0 < 0.3 low level of evenness of species
E = 0.3 < 0.6 medium level of evenness
E = > 0.6 high level of species evenness

e. Species Richness Index

Species Richness Index is a measure of the diversity of plant species found in a location. Species richness refers to the number of individuals of each species found in a location or community. The more species there are, the greater the richness index. According toAnjani et al. (2022), species richness is calculated using the Margalief index, namely:

$$\mathbf{RI = \frac{(S-1)}{In\ (N)}}$$

where:
Republic of Indonesia: Margalief Wealth Index
S: Number of Species Found
N: Total Number of Individuals

The categories for determining species wealth for the Margalef Wealth Index are as follows:

Dmg = < 3.5 low type wealth naka
Dmg = 3.5 - 5 then the wealth of the type is moderate
Dmg = > 5 then the richness of the high type

A. Soybean Seed Sampling

The soybean seed samples used were primary samples that entered the BKHIT laboratory in October 2024. The packaged samples have been labeled with information such as archive number, sample code, sample type, and archive date. Referring to the International Seed Testing Association (ISTA) in 2024, the minimum weight of the soybean seed sample taken is 1000 g, with a minimum number of test samples of 400 grains. Soybean seeds were taken from primary samples for testing using the

probability sampling method, while samples for incubation were taken using the simple random sampling method.

B. Literature Study

Literature study was conducted to find supporting data for research such as soybean export procedures from the United States, location conditions such as geography and climate of the sample location being cultivated, specifications of the transport ship, and conditions of the ship's cargo during the voyage as well as supporting factors for soybean cultivation in the United States. Literature study was conducted to find information related to the category of types of fungi found with the Determination Key from Sine & Soetarto (2018).

C. Data analysis

The data obtained from the study were processed using Microsoft Excel. Meanwhile, the Analysis of Variance (ANOVA) test was carried out at a level of 5%. Further testing was carried out using the Duncan's Multiple Range Test (DMRT) method with additional software, namely DSAASTAT ver. 1.514

RESULTS AND DISCUSSION

Based on the results in Table 2, two types of colonies were found with different characteristics that were adjusted to the morphological characteristics according to(Sine & Soetarto, 2018),It is known that in Colony 1, the spores are black, the mycelium is white, the columella and spores are globose, the colony texture is filamentous, the hyphae are not septate, and the spore head is globose so it is identified as Rhizopus sp. Furthermore, in Colony 2, the spores are green, the mycelium is white, the columella and spores are globose, the colony texture is filamentous, the hyphae are not septate, and the spore head is globose, so it is identified as *Aspergillus* sp..

Tabel 1. Persentase eksplan terkontaminasi, browning dan steril

Character	Colony 1	Colony 2
Spore color	Black	Green
Mycelium color	White	White
Columella shape	*Globose*	*Globose*
Spore form	*Globose*	*Globose*
Colony texture	*Filamentous*	*Filamentous*
Types of hyphae	No partition	No partition
Spore head shape	*Globose*	*Globose*
Identified as:	*Rhizopus*sp.	*Aspergillus*sp.

Description: Colonies are distinguished based on differences in colony color during direct observation and under a microscope.

1. *Rhizopus* sp.

The fungus found in Colony 1 (Figure 1) was identified as Rhizopus sp. This fungus has characteristics with black spores, white mycelium, smooth-textured sporangiophores, globose or round columella and spores, rhizoids that appear opposite to the direction of the sporangiophores, round colonies with non-septate hyphae, and round spore heads. Rhizopus sp. fungi have very fast growth and within 5 days will spread throughout the surface of the Petri dish along with the growth of stolons.

Rhizopus sp. spores are in the form of Oidia, which are asexual spores and are formed due to the breaking of hyphae when the sample is tested with the aid of a microscope. These characteristics are in line withSine & Soetarto (2018), where the sporangiophores formed are usually in groups of two, three, or more, but can also be just one. Sporangia are the same shape, round or almost round, with a slightly flat center.

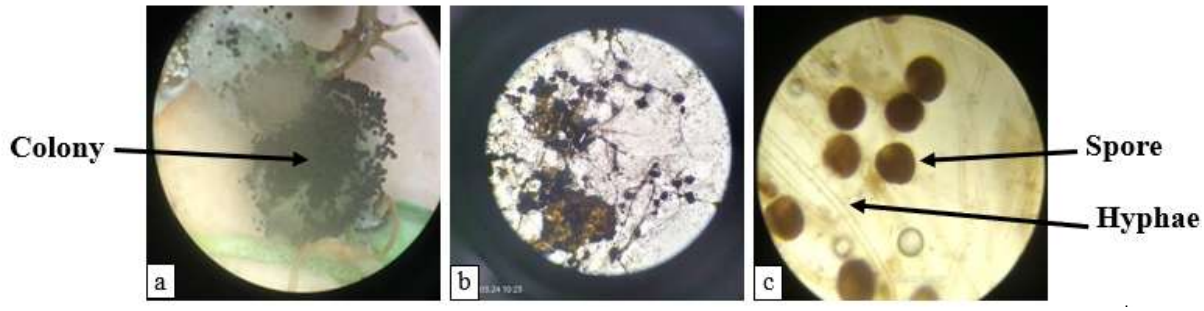

Figure 1. Rhizopus sp. colony. a) Stereo microscope lens 10x magnification, b) 10x magnification compound microscope, c) 40x magnification compound microscope

2. *Aspergillus*sp.

The fungus found in Colony 2 (Figure 2) was identified as Aspergillus sp. This fungus has characteristics with dark green spores, white mycelium, smooth-textured sporangiophores, globose or round columella and spores and asexual spores, rhizoids that appear opposite to the direction of the sporangiophores, round colonies with aseptate or non-septate hyphae, and round spore heads. Aspergillus sp. is a genus of filamentous fungi that are widespread in various environments, including soil, air, and organic materials such as grains and plants. This fungus belongs to the Ascomycota group and is known for its ability to grow on substrates with low water content, making it one of the main contaminants in agricultural products, including soybeans.

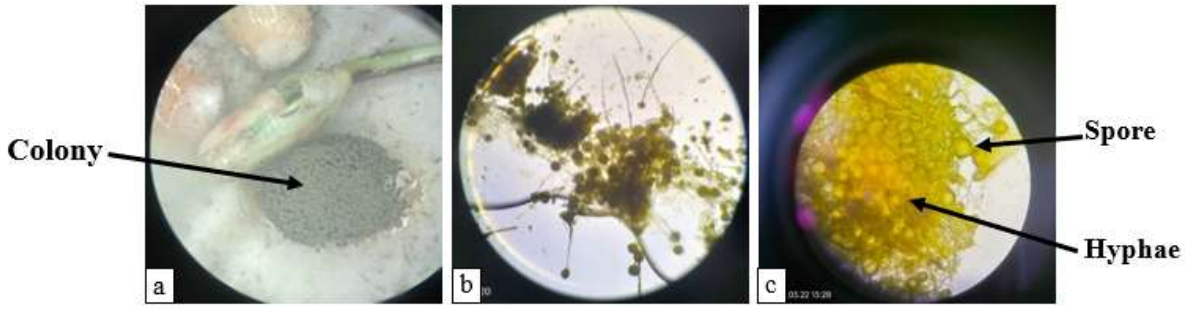

Figure 2. Aspergillus sp colony a) Stereo microscope lens 10x magnification, b) 10x magnification compound microscope, c) 40x magnification compound microscope

Aspergillus sp. is a genus of filamentous fungi that are widespread in various environments, including soil, air, and organic matter such as grains and plants. This fungus belongs to the Ascomycota group and is known for its ability to grow on substrates with low water content, making it one of the main contaminants of agricultural products, including soybeans. Most fungi require high relative humidity and temperature to grow and develop. Its development increases in microclimate environments caused by condensation, but some species of fungi can live at low water activity, so that they are classified as xerophilic fungi. Aspergillus sp. is a genus of fungi that can produce aflatoxin compounds, which can be harmful to the health of plants and their derivative products that are processed into processed foods. This is in line with Nino & Neonbeni (2020), that this compound can cause liver dysfunction to the point of causing hepatotoxicity with symptoms such as fever, vomiting, abdominal pain, anorexia, and damage to the immune system.

It is known that the results in Table 3 show that filter paper does not have a significant effect on the growth of Rhizopus sp. fungi, but has the highest frequency of

Rhizopus sp. findings. It is also known that filter paper is a substrate with the highest fungal findings of six colonies in repetitions two and four, which shows that this type of substrate is more effective than other types of substrates, although the percentage of findings is relatively very small, namely below one percent.

Based on the results presented in Figure 3, it was found that filter paper can store moisture better than opaque paper and kitchen tissue. The large number of Rhizopus sp. colonies found on the filter paper substrate is in line withGoodell et al. (2020), where the structure of filter paper is generally made from 40% cellulose and is ash-free, so that the response of fungi to the pentose and hexose sugars in it is to make it a source of energy to support the growth of fungi such as Rhizopus sp.

Table 3. Results of the Calculation of the Frequency of Findings of Rhizopus sp. Fungus

Substrat	Test												Average
	U1	%	U2	%	U3	%	U4	%	U5	%	U6	%	
KS	5	0.61	6	0.74	5	0.61	6	0.74	0	0	0	0	3.6a
KB	1	0.12	1	0.12	1	0.12	1	0.12	2	0.24	1	0.12	1.1a
TD	0	0	2	0.24	1	0.12	1	0.12	1	0.12	1	0.12	1a
Average	6		3		2.3		2.6		1		0.6		

Description: Numbers followed by the same letter in the same column show no significant difference based on the 5% DMRT test

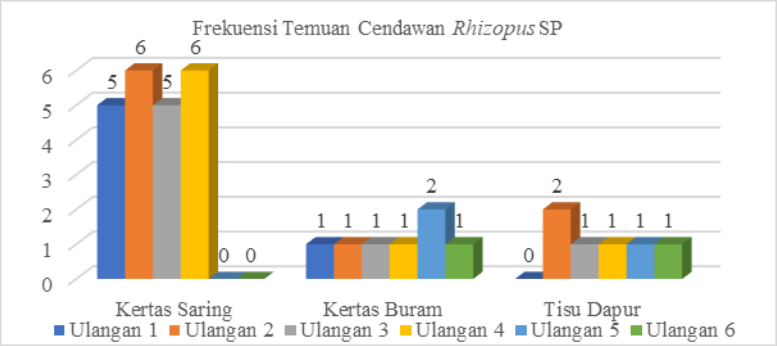

Figure 3. Frequency Graph of Findings of Rhizopus SP Fungi

Based on the results in Table 4, it is known that filter paper has a significant effect on the growth of Aspergillus sp. fungi, and has the highest frequency of Aspergillus sp. findings. Filter paper is the substrate with the highest frequency of findings, reaching 38 seeds in repetition 5 and 31 seeds in repetition 6. Similar things were also found on the opaque paper and kitchen tissue substrates, where repetitions 5 and 6 had the highest frequency of findings, each of which was 22 seeds and 21 seeds on the opaque paper substrate and 20 seeds and 25 seeds on the kitchen tissue substrate. It is known that filter paper also has the highest average in the discovery of Aspergillus sp. fungi, with a value of 17 colonies.

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Table 4. Results of the Calculation of the Frequency of Findings of Aspergillus sp. Fungus.

Substrat	Test												Average
	U1	%	U2	%	U3	%	U4	%	U5	%	U6	%	
KS	11	0.61	9	0.74	7	0.61	8	0.74	38	0	31	0	17.3a
KB	2	0.12	3	0.12	1	0.12	6	0.12	22	0.24	21	0.12	9.16b
TD	1	0	2	0.24	6	0.12	4	0.12	20	0.12	25	0.12	9.67b
Average	4.6		4.6		4.6		6		26.6		25.6		20.57

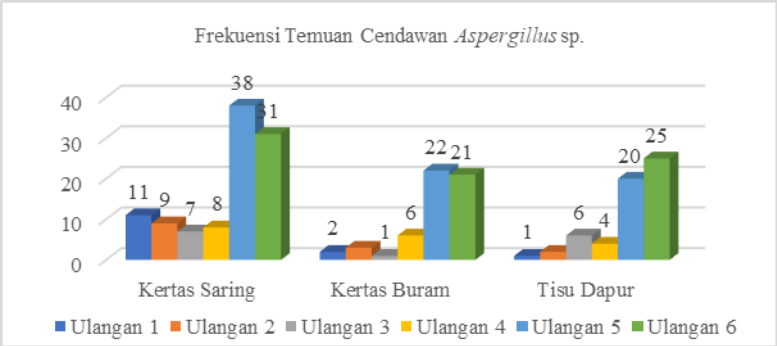

Figure 4. Frequency Graph of Findings of Aspergillus sp. Fungus

Based on the results in Table 4, it is known that the frequency of findings of Aspergillus sp. fungi has the highest percentage of findings compared to Rhizopus sp. fungi in soybean seeds incubated using the three types of substrates. The total percentage of frequency of findings of fungi shows that Aspergillus has the highest percentage, reaching 13.08% on filter paper, 6.79% on opaque paper, and 7.16% on kitchen tissue. The high frequency of findings of Aspergillus sp. is in line withJoseph (2022), that some conditions that affect the growth of this fungus, such as high humidity and minimal direct sunlight. Based on the results in Table 5, the results of the percentage frequency of fungal findings with filter paper as the substrate with the highest total percentage of 22.59%, followed by opaque paper with a percentage of 15.06%, and kitchen tissue with 14.07%.

According to Sulistiani & Isworo (2022) Filter paper has special characteristics that make it ideal for all types of seed testing which in this case is seen from the results of post-research incubation. Characteristics such as pore diameter affect its porosity in maintaining its moisture. This makes filter paper able to store moisture better than opaque paper and kitchen tissue. The large number of colonies found on the filter paper substrate is in line withHu et al. (2020), where the plant structure is composed of cellulose and hemicellulose, the fungal response to pentose and hexose sugars was tested to determine whether these substrates could support fungal growth. All carbohydrate sources supported the growth of Rhizopus sp.

Based on the results of the calculation of the fungal diversity index in Table 6, it is known that the filter paper substrate has the highest results, namely 0.59 in the growth of the two types of fungi identified, although its diversity is relatively low. The dominant growth of Aspergillus sp fungi can be caused by homogeneous environmental conditions because the study was conducted in a laboratory. This is supported by Saputra et al. (2024), where a community can be very diverse if many different and abundant species are found. Conversely, a community with few species identified or found will have low diversity.

Based on the results of the species evenness index in Table 5, it is known that

the three substrates have the same value, namely above the range of 0.3, which means that the level of species evenness of each substrate is classified as moderate. However, filter paper is the substrate with the highest evenness index, reaching 0.52 due to the large number of colonies found. Based on the results in Table 7, it is known that the value of the species richness index in the filter paper treatment is slightly smaller than the opaque paper and filter paper treatments. This shows that a greater number of individuals were found on filter paper, but the number of species found in the three substrates was relatively the same.

Table 5. Frequency of Fungal Findings

Petri Dish	Substrate Treatment					
	Filter Paper	(%)	Opaque Paper	(%)	Kitchen Tissue	(%)
*Rhizopus*sp.	22	2.71	6	0.037	6	0.74
*Aspergillus*sp.	106	13.08	55	6.79	58	7.16
Total	183	22.59	122	15.06	114	14.07

Table 6. Results of Species Diversity Index Calculation

Substrat	Fungus	Amount	Pi	InPi	PilnPi
Paper	*Rhizopus*sp	22	0.08	-2.43	-0.21
Filter	*Aspergillus*sp	104	0.41	-0.88	-0.36
H'		126	0.49	-(-3.32)	-(-0.59)
Paper	*Rhizopus*sp	7	0.02	-3.58	-0.09
Opaque	*Aspergillus*sp	55	0.21	-1.52	-0.33
H'		62	0.23	-(-5)	(-0.42)
Tissue	*Rhizopus*sp	6	0.02	-3.73	-0.08
Kitchen	*Aspergillus*sp	58	0.23	-1.46	-0.33
H'		252	0.25	-(-5.19)	-(-0.41)

Information H' = < 1, including low-level diversity
H' = 1-3 Includes a moderate level of diversity
H' = > 3, including a high level of diversity

Table 7. Diversity, Evenness, and Species Richness Indexes in Tested Substrates

Treatment	Number of Species	Diversity Index	Equity Index	Species Richness Index
Filter Paper	2	0.28	0.25	0.56
Opaque Paper	2	0.61	0.56	0.41
Kitchen Tissue	2	0.58	0.53	0.42

The low evenness index results were caused by the presence of one species of fungus with dominant growth on all tested substrates, namely Aspergillus sp. This is in line with Setiarno et al. (2022), where the more evenly distributed a species is in a research location, the higher the evenness index value will be. Conversely, if there is a species that dominates a research location, it will produce a low evenness index value. The results of the richness index also show a low number, namely below 3.5. The low figure is according to Asrianny et al. (2019), a correlation between the value of the species richness index and the species found in a place or community. The more species of organisms found, the greater the value of the richness index. Species that are abundant in a location generally have a small number of individuals per species. However, this will affect the value of the richness index. The small number of fungal species found from the three substrates was due to the homogeneous environmental conditions of the study, so that the potential for various factors that affect the growth of

various types of fungi on soybean seeds can be minimized

CONCLUSION

Based on the research results, two types of fungi were identified in soybean seeds from the United States through blotter testing using different substrate variations based on morphological characteristics, namely Rhizopus sp and Aspergillus sp, with Aspergillus sp. as the fungus with the highest frequency of findings. The application of the blotter method using filter paper gave the best results in identifying fungi based on the frequency of fungal findings. The presence of both types of fungi indicates that soybeans are dangerous for consumption because they contain Aflatoxin compounds. It is recommended to use filter paper in detecting the presence of fungi in soybean seeds and combined with other methods such as Nutrient Agar (NA) or Potato Dextrose Agar (PDA) medium.

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