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Isolation of *Neurospora* and its interaction with maize

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Abstract

Neurospora crassa is commonly known as red bread mould as it produces luxuriant and clearly visible orange spores (conidia). *Neurospora* possesses a combination of features which makes it an ideal organism for research. It is easy to grow and maintain in the laboratory, has short life cycle and it is haploid that makes genetic analysis simple. Most importantly *Neurospora* is non-pathogenic and it is even used as food in many countries. *Neurospora* has been observed in many countries, growing commonly on vegetation scorched by fire, on corn cobs and in sugar cane fields. In India and particularly in Ujjain, *Neurospora intermedia* strains has been found growing on discarded corn cobs regularly. Although *Neurospora* is well established saprophytic soil borne fungus but recent studies indicate that it may live in some plants as endophytic fungus. Efforts are needed in this area to confirm these results and to study the interaction of *Neurospora* with plants which are natural substrate for this fungus like corn and sugarcane. In view of this it was decided to study interaction of *Neurospora* with maize during this study.

Keywords: Bread mould; Conidia; Corn; Endophytic fungus; *Neurospora;* Saprophytic

1. Introduction

Neurospora is a well-known model organism for studies of biochemistry and genetics [1,2]. It is an ideal organism for research, because of its haploid nature, short life cycle and minimal growth requirements [3]. It is a filamentous fungus and it is also being used by several researchers for understanding growth and morphogenesis of fungi [4,5]. *Neurospora* is an ascomycete fungus and is abundantly found on burnt vegetation like sugarcane and discarded corncobs in tropical and subtropical regions [6,7,8]. There are five conidiating species of *Neurospora* which are *N. intermedia, N. crassa, N. tetrasperma, N. discreta* and *N. sitophila,* but in nature *N. intermedia* is most frequently found. All species have been reported from India including yellow ecotypes and orange ecotypes of *N. intermedia* [6,7]. Although, *Neurospora* is a well-known saprophytic fungus but recently several studies have shown that it also has endophytic lifestyle [9].

Fang *et al*., [10], studied the endophytes in leaves and stems of *Camellia sinensis* and they found *Neurospora crassa* in leaves and stems of this plant along with nine other fungi in all the seasons. Qi *et al*., [11] studied the endophytic fungi associated with *Acer ginnala* in China and they also reported that *Neurospora* sp. was dominant endophyte in this plant. Sudarma *et al*., [12] reported the presence of *Neurospora* sp. as exophytic as well as endophytic fungus in *Annona squamosa* L.

It has been suggested that lifestyle of endophyte is controlled by both environment and host factors and by switching between endophytic, saprotrophic and pathogenic lifestyles the fungus can adjust to changing [9,13,14,15].

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The aim of the study was to isolate *Neurospora* from discarded corn cobs along with the study of interaction of *Neurospora* with maize. The effect of seed/ soil inoculation with spores of *Neurospora* on seed germination, seedling growth and plant growth of maize was also studied, so that it may be used as potential ecofriendly bioinoculants/ biofertilizers.

2. Materials and Methods

2.1. Sample collection

Visible conidia of *Neurospora* were collected from Ujjain Railway station by following the modified method described by Perkins and Turner [8]. A test tubes containing filter paper strip and tooth pick were sterilized and taken to sampling site and visible conidia of *Neurospora* were collected on sterilized filter paper strip and bought to the laboratory (Figure 1).

A B

Figure 1 Collection of *Neurospora* conidia from discarded corn cobs

2.2. Isolation and purification of *Neurospora intermedia*

Filter paper strips containing *Neurospora* conidia were placed on Vogel's medium containing Petri plates supplemented with 0.02% chloramphenicol and incubated at 34 ± 2 °C. After 24 hours the conidia on filter paper germinated and formed conidiating colony of *Neurospora* (Figure 2). Small amount of conidia were transferred to Vogel's medium slants and incubated at 34 ± 2 °C to obtain profusely conidiating *Neurospora* cultures. Repeated subculturing was done for purification of cultures.

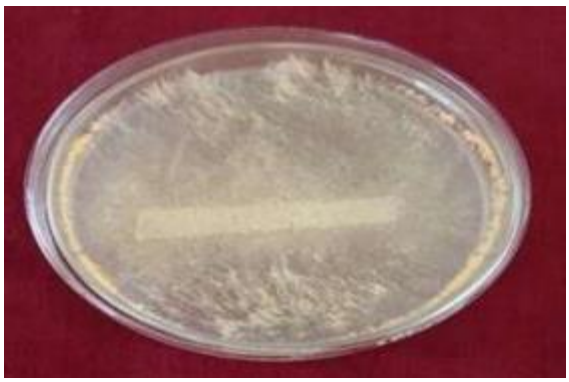

Figure 2 Isolation of *Neurospora* from conidial samples collected on filter paper strips

2.3. Identification of *Neurospora* cultures

Synthetic crossing (SC) medium was used for making genetic crosses for determination of mating types and identification of species of isolated *Neurospora* cultures. The SC medium was prepared by adding stock solution of SC medium and distilled water in 1:1 ratio, 1.5% agar and 1% sucrose [16].

2.3.1. Mating type and species determination of Neurospora strains

Mating type of wild-type *Neurospora* cultures were determined by making spot crosses as described by Perkins *et al*., [17]. Lawns of fluffy cultures of both mating types of *N. crassa* were created on SC medium Petri plates by inoculating the cultures and incubating at 25 °C for 7 days. A small quantity of conidia of isolated *Neurospora* culture was rubbed over the fluffy lawn and incubated for 2 days at 25 °C and mating type reaction was observed. If well-formed black perithecia developed then the culture had mating type opposite to that of the fluffy culture used for making lawn and if no perithecia developed then the mating type of culture was similar to the mating type of fluffy culture used for making lawn (Figure 3).

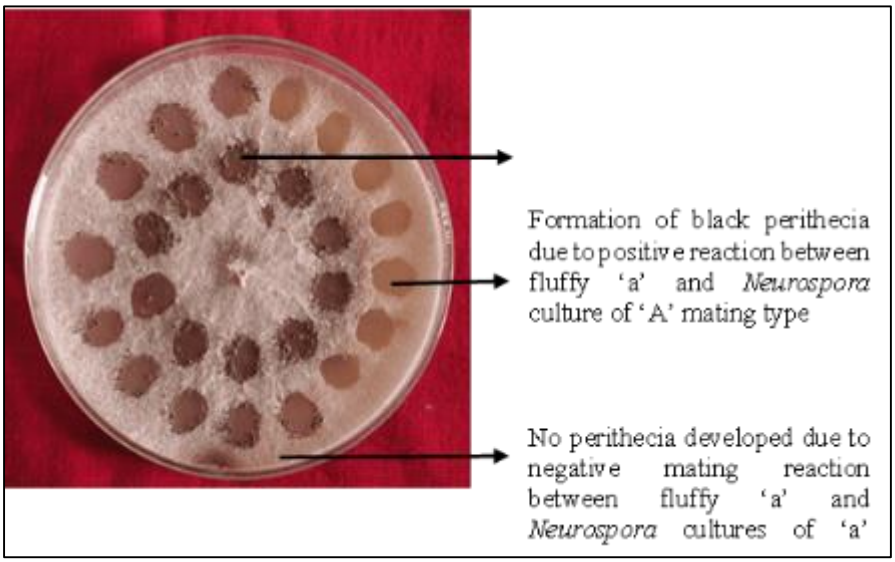

Figure 3 Determination of mating type of *Neurospora* cultures

2.4. Identification of species of *Neurospora* cultures

The identification of species of *Neurospora* cultures was done by testing the fertility in crosses with different tester strains as recommended by Perkins and Turner [8]. Genetic crosses of isolated *Neurospora* cultures were made with tester strains of different species of *Neurospora* obtained from FGSC (Fungal Genetics Stock Center), School of Biological Sciences, University of Missouri, Kansas City, USA (Table 1). In order to make cross the tester strain was used as female parents and inoculated on SC medium tube and incubated at 25 °C for 7 days. The conidia of isolated culture of opposite mating type were rubbed over the tester strains on SC medium and the cross tube was incubated at 25 °C for 10 days. When a culture was crossed with tester strain of same species then it produced abundant black perithecia and ascospores. However, when the culture was crossed with tester strain of different species then rudimentary perithecia developed and few ascospores were formed. The isolated *Neurospora* cultures were crossed with *N. crassa* (FGSC # 2489/ FGSC # 4200), *N. discrete* (FGSC # 3228/ FGSC # 3229), *N. intermedia* (FGSC # 1766/ FGSC # 1767) and *N. sitophila* (FGSC # 2216/ FGSC # 2217).

The colour of the conidia of the cultures was visually recorded and conidial size was determined using micrometer to identify different ecotypes on *Neurospora intermedia*.

Table 1 Tester strains used for identification of species of *Neurospora* cultures

S. No.	Species	FGSC No.	Mating type
1	*Neurospora crassa*	FGSC # 2489 FGSC # 4200	A A
2	*Neurospora intermedia*	FGSC # 1766 FGSC # 1767	A A
3	*Neurospora discreta*	FGSC # 3228 FGSC # 3229	A A
4	*Neurospora sitophila*	FGSC # 2216 FGSC # 2217	A A

2.5. Effect of selected *Neurospora* strain (N1) on seed germination and seedling growth of maize

Culture N 1 was selected for studying its effect on seed germination and seedling growth of maize. This culture produced profuse yellow coloured conidia and its mating type was 'A'.

The fungal cultures was grown in 250 ml conical flask having 50 ml Vogel's medium, for 7-10 days at 28 ± 2 °C or 34 ± 2 °C. The spores were harvested using the method described by Elias *et al.,* [18]and a final fungal spore suspension having 10^5-10^6 spores/ml was prepared. The spore suspension was used on the same day on which it was prepared.

Dry maize seeds (variety MRM 3777) were purchased from local shop of Ujjain and surface sterilized as described by Han *et al*., [19]. After sterilization maize seeds were immersed in fungal spore suspension for 24 h and for control maize seeds were immersed in sterile distilled water. After spore treatment seeds were washed with sterile distilled water and placed on wet cotton containing Petri plates. In each Petri plates three seeds were placed and incubated at 28 ± 2 °C. The plates were monitored regularly and if required more sterile distilled water was added. Germination percentage was calculated after 24-48 h incubation by following formula [20]:

$$\textbf{Seed germination percentage} = \frac{\textbf{Number of seeds germinated x 100}}{\textbf{Total number of planted seeds}}$$

The seedling growth was observed on 5^{th} day and root length, shoot length was measured. The roots and shoots were wrapped in aluminium foil and kept in hot air oven for 2 h at 80 °C after which their dry weights were measured [21]. Vigour index was calculated by using formula given below [22]:

Vigour index = Seed germination (%) x (Mean shoot length + Mean root length)

2.6. Pot culture experiments

The pot culture experiments were done in 1 L polypropylene autoclavable beakers having capacity of containing 1 Kg soil. The experiments were performed using sterilized soil. The sterilized soil was used so that only the effect of inoculated fungal cultures could be observed. The soil was sterilized three times with an interval of 24 hours between each cycle.

Maize seeds were surface sterilized by the method described by Han *et al*., [18] and seeds were placed 2 cm deep under the sterilized soil. In each pot 6 seeds were placed at equal distance and spore suspension was added on the seeds and the seeds were covered with layer of soil. Water was added to the pots till the soil become moist. The pots were kept in natural conditions and in natural light and more water was added later on according to weather conditions. After 7 days plants were harvested and shoot growth (shoot length/ plant height, area of largest leaf, dry weights of shoots), root growth (root length and dry weights of roots) were measured. The plant parts (shoots/ roots) were wrapped in aluminium foil and kept in hot air oven for 24 h at 80 °C after which their dry weights were recorded [23,24].

The experiment was performed in triplicate.

2.7. Statistical analysis

The experiment was performed in triplicate and the data were subjected to analysis of variance (ANOVA) using VassarStats. The mean and standard deviation of data were compared using Tukey's HSD test at $P \leq 0.01$ and $P \leq 0.05$.

3. Results

3.1. Isolation and Identification of *Neurospora*

Twenty *Neurospora* cultures were isolated from conidial samples collected during the study. The mating types and species of *Neurospora* cultures were determined and results are shown in Table 2 and Figure 4.

The results show that all the twenty cultures belonged to *Neurospora intermedia* and had large sized (11-19 µm) yellow coloured conidia (Figure 4). It was further found that 12 cultures of yellow ecotype had mating type *'A'* while, 8 cultures of yellow ecotype had mating type *'a'*. Thus, it appears that 60% cultures had *'A'* mating type and 40% cultures had *'a'* mating type. The almost equal frequency of *'A'* and *'a'* mating types in isolated cultures indicates that *Neurospora* at this place is sexually reproducing.

Table 2 *Neurospora* cultures isolated during the study

S. No.	Culture Number	Place of collection	Colour of Conidia	Mating type	Species
1	N 1	Madhav nagar Railway station road	Yellow	A	*N. intermedia*
2	N 2	Madhav nagar Railway station road	Yellow	a	*N. intermedia*
3	N 3	Madhav nagar Railway station road	Yellow	a	*N. intermedia*
4	N 4	Madhav nagar Railway station road	Yellow	a	*N. intermedia*
5	N 5	Madhav nagar Railway station road	Yellow	a	*N. intermedia*
6	N 6	Madhav nagar Railway station road	Yellow	a	*N. intermedia*
7	N 7	Ujjain Railway station track-7	Yellow	a	*N. intermedia*
8	N 8	Ujjain Railway station track-7	Yellow	a	*N. intermedia*
9	N 9	Ujjain Railway station track-7	Yellow	a	*N. intermedia*
10	N 10	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
11	N 11	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
12	N 12	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
13	N 13	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
14	N 14	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
15	N 15	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
16	N 16	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
17	N 17	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
18	N 18	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
19	N 19	Ujjain Railway station track-7	Yellow	A	*N. intermedia*
20	N 20	Ujjain Railway station track-7	Yellow	A	*N. intermedia*

Figure 4 Yellow ecotype of *Neurospora* intermedia cultures isolated during the study

3.2. Effect of N 1 (*Neurospora intermedia)* on seed germination, seedling growth and vigour index

The spore suspension of *Neurospora* culture (N 1) containing 10^5-10^6 spores/ml was used for seed treatment and control seeds were treated with sterile distilled water. The germination percentage of seeds was observed after 24-48 hours. It was seen that seeds treated with sterile distilled water as well as fungal spores demonstrated 100% germination (Table 3). This indicates that this fungus does not have any negative effect on germination of seeds when treated with this concentration of spores. It was also seen that using spore suspension above this concentration resulted in damage of seeds.

Table 3 Effect of N 1 fungi on seed germination

S. No.	Seed treatment	Seed germination (%)
1	Control	100%
2	N 1 (*Neurospora intermedia*)	100%

The effect of fungal treatment on shoot length is shown in Figure 5 and Table 4. It can be seen that there is 52.90% increase in shoot length when treated with N 1. The results show that there is significant increase in shoot length in maize seeds when treated with fungal spores of N 1. The mean dry weight of shoots of three maize seeds in control seeds was 0.673 ± 0.005 mg and the mean dry weights of shoots of seeds treated with N 1 was 0.867 ± 0.005 mg. The dry weights of shoots of maize seeds also showed significance increase when treated with N 1 spore suspensions.

The effect of fungal treatment on root length is shown in Figure 5 and Table 4. It can be seen that there 49.64% increase in root length when treated with N 1. The results reveals that there is significant enhancement in root length of maize when seeds are treated with N 1 spore suspensions. The mean dry weight of roots of three maize seeds in control seeds was 0.087 ± 0.002 mg and the mean dry weight of roots of seeds treated with spores of N 1 0.147 ± 0.003 mg. The dry weight of roots of seedlings also shows significant increase by treatment with spores of N 1. Figure 5 shows that there is increase in lateral roots and root hairs by N 1 treatment of seeds in comparison to control.

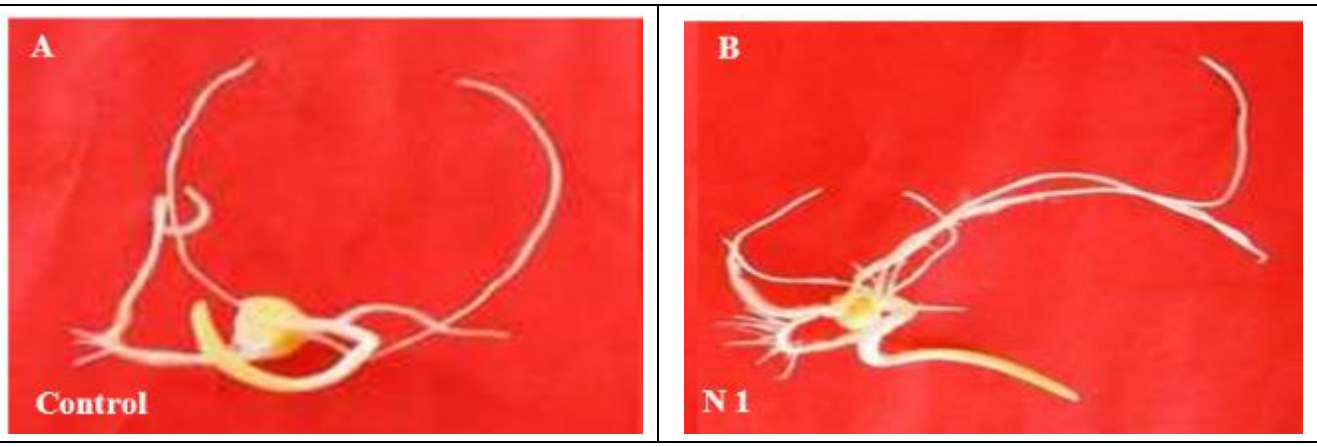

Figure 5 Effect of N 1 fungal spore suspension on seedling growth of maize. (A) Seeds treated with distilled water. (B) Seeds treated with spores of N 1

Table 4 Effect of N 1 spores on shoot growth and root growth

S. No.	Seed treatment	Shoot growth		Root growth	
		Shoot length (cm)	Dry weight of shoots (mg/3 seeds)	Root length (cm)	Dry weight of roots (mg/3 seeds)
1	Control	9.13 ± 0.41	0.673 ± 0.005	9.73 ± 0.30	0.087 ± 0.002
2	N 1	13.96± 0.30* (52.90)	0.867 ± 0.005* (28.82)	14.56 ± 0.37* (49.64)	0.147 ± 0.003* (68.96)

Each value is represented in mean ± SD with three replicates and values in parenthesis indicate percentage increase in growth parameters.

*Denote significant difference at $P < 0.01$ between control and treated seeds within a column.

It was seen that the seedling vigour also increased when treated with fungal spore suspension. The vigour index of control seeds was 1886 ± 3 and the vigour index of seeds treated with N 1 was 2863 ± 6 (Table 5). These results demonstrate that treatment of maize seeds with fungal spores significantly enhances the seedling growth and vigour of seedlings.

Table 5 Effect of N 1 spores on seedling vigour

S. No.	Seed treatment	Vigour index
1	Control	1886 ± 3
2	N 1	2863 ± 6* (51.80)

Each value is represented in mean ± SD with three replicates and values in parenthesis indicate percentage increase in vigour index.

*Denote significant difference at $P < 0.01$ between control and treated seeds within the column.

3.3. Effect of fungal spores N 1 on maize plants grown in sterilized soil in pot culture experiment

In pot/ beaker containing sterilized soil 5 ml/seed fungal spore suspension of *Neurospora intermedia* (N 1) was added. In control experiments distilled water was added in place of spore suspension. The effect of spores on shoot growth (shoot length/ plant height, area of largest leaf and dry weights of shoots) and root growth (root length and dry weights of roots) was studied after 7 days of growth (Figure 6).

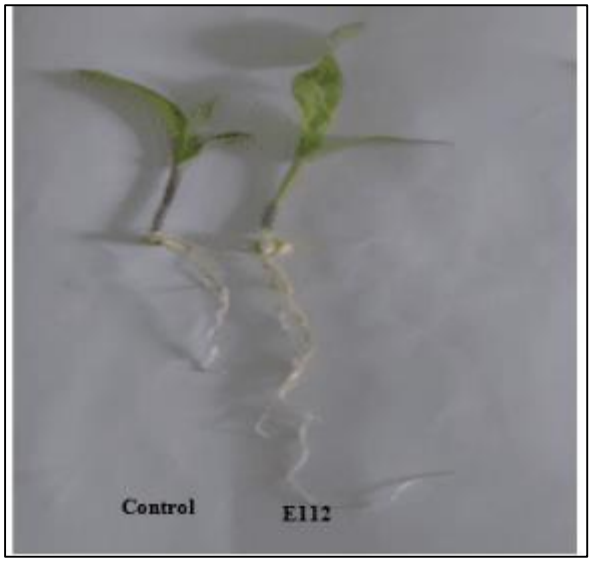

Figure 6 Effect of N 1 on growth of maize plants in sterilized soil. The photograph shows comparison of control plants (C) and plants grown in soil supplemented with N 1 fungal spores

The effect of N 1 fungal spore suspension on shoot length/ plant height is shown in Table 6. It can be seen that addition of 5 ml/seed fungal spore suspension of N 1 significantly increases the shoot length in comparison to control plants. The addition of 5 ml/seed concentration of spores of N 1 resulted in 39.43% increase in shoot length, 45.77% increase in root length and 64.83% increase in leaf area (Table 6).

Table 6 Effect of N 1 on shoot length, root length and leaf area of maize plants grown in sterilized soil

S. No.	Treatment	Shoot length (cm)		Root length (cm)		Leaf area (cm^2)	
		Control (dH_2O)	N 1 (Spore suspension)	Control (dH_2O)	N 1 (Spore suspension)	Control (dH_2O)	N 1 (Spore suspension)
1.	5 ml/seed	13.72 ± 1.85	19.13 ± 3.00* (39.43)	14.66 ± 1.52	21.37 ± 1.90* (45.77)	81.9 ± 6.6	135.0 ± 13.0* (64.83)

Each value is represented in mean ± SD with three replicates and values in parenthesis indicate percentage increase in shoot/root length and leaf area; *Denote significant difference at $P < 0.01$ between control and treated plants within a row; Values within columns are not significantly different.

4. Discussion

Neurospora is a well-known saprophytic fungus but recently, it has been shown that *Neurospora* can also live as endophyte in Scots pine (*Pinus sylvestris*), *Camellia sinensis*, sugar apple (*Annona squamosa* L.) and *Acer ginnala* Maxim. [9,10,11,12]. Studies by Pandit and Maheshwari, [6] have shown the presence of *Neurospora intermedia* growing on sugarcane stumps. Although they did not consider *Neurospora* as an endophytic fungus but they clearly demonstrated the presence of *Neurospora* mycelium below the epidermis of sugarcane stumps in the sugarcane field. Profusely conidiating yellow ecotype and orange ecotype of *Neurospora intermedia* have been regularly observed on discarded corn cobs in Ujjain Railway station and Sanver road [7,25]. It appears that *Neurospora* may infect the discarded corn cobs by soil borne spores of *Neurospora*, however, more extensive sampling and isolation from maize tissues need to be carried out in future to conclusively determine the presence/ absence of *Neurospora* as endophyte in maize. The presence of almost equal frequencies of '*A*' and '*a*' mating types in natural population of *Neurospora* implies that *Neurospora* reproduces sexually as suggest in earlier studies [7]. was selected for studying interaction of *Neurospora* with maize which is its natural substrate [7,25] as recent studies have implied that it also has endophytic life style in some plants [9,10,11,12]. *Neurospora intermedia* was selected for studying interaction of *Neurospora* with maize which is its natural substrate [7,25] as recent studies have implied that it also has endophytic life style in some plants [9,10,11,12]. It was seen that *Neurospora intermedia* (N 1) had no negative effect on seed germination in maize, if spore suspension was added in optimal concentration. The shoot length, root length, shoot biomass and root biomass of maize

seedlings significantly increased in comparison to control and the seedling vigour was enhanced by treatment of maize seeds with spore suspension of this fungus.

Vitorino *et al*., [26] found that seedlings of *Eucalyptus grandis* and *Eucalyptus urophylla* hybrids were stimulated by inoculation with endophytic fungi *Fusarium* sp., *Papulaspora* sp. and *Trichoderma* sp. and there was positive effect on stem length, stem diameter, fresh biomass and dry biomass of treated seedlings. Kedar *et al*., [22] reported the growth promotional potential of two *Phoma* sp. on maize. It was observed that fungal inoculation increases germination of seeds and there was increase in shoot length and root length in comparison to control seeds. It was also seen that roots of treated seeds developed profuse root hairs. They also suggested that these roots hair may help in absorbing more nutrients for increasing growth of plants. It has also been suggested that endophytic fungi cause degradation of cellulose in cuticle in seed germination and provide carbon for growth of seedlings which results in increase in seed germination and seedling [23].

The spore suspension of *Neurospora intermedia* (N 1) was inoculated in sterilized soil. It was observed that N 1 culture positively influenced the growth of maize plants in sterilized soil. There was significant increase in plant height/ shoot length, root length, leaf area and biomass of plants grown in sterilized soil inoculated with fungal spores. The results of this study (Figure 6) also suggest that there is considerable increase in lateral roots and root hairs by treatment with these fungi. Abdel-Motaal et al., [27] found that treatment of tomato seeds with *Aspergillus flavus* causes early emergence of seedlings and enhances germination percentage, fresh weight and seedling length over the control. Naziya *et al*., [28] have also demonstrated that treatment of chilli seeds with isolates of *Aspergillus* sp., *Penicillium* sp., *Talaromyces* sp. and *Trichoderma* sp. enhanced seed germination and seedling vigour significantly. The seed treatment with *Talaromyces* sp. resulted in 86.25% increase in seed germination and enhancement in seedling vigour.

5. Conclusion

During this study 20 cultures of yellow ecotype of *Neurospora intermedia* were isolated from discarded corn cobs and identified. Effect of selected culture N 1 on seed germination, seedling growth and plant growth in pot cultures was studied. The result show that this culture has no negative effect on seed germination, seedling growth and plant growth of maize. Further, it appears that use of fungal spores of this culture as bioinoculant slightly increases seedling growth, seedling vigour and plant growth. Thus, result of this study points out that *Neurospora* has no negative interaction with maize and presence of *Neurospora* in fields has positive effect on seed germination and growth of maize plant.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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