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AN INVESTIGATION OF BIODIVERSITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH SOME MEDICINAL PLANTS

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Abstract: The present investigations of Endophytic fungi residing in medicinal plants have not been systematically characterized. In this study, we isolates from 5 medicinal plant species using traditional morphological methods. The colonization rate, isolation rate, and relative frequency of these endophytes were investigated. The relationship between the composition of endophytic fungi and the chemical constituents of host plants was also explored for the first time. The results showed that endophytic fungi from these medicinal plants exhibited high biodiversity, host-recurrence, tissue-specificity, and spatial heterogeneity. Taxa of *Colletotrichum spp, Aspergillus spp, Bispora spp., Geotrichum spp., Trichoderma spp. Cladosporium spp. Aspergillus spp. Gliocladium spp., Trichoderma spp. Fusarium spp. Trichosporoniodes spp Trichoderma spp* and mycelia sterilia were the dominant fungal endophytes. Some Aliphatic, Aromatic, Amines, Aldehyde, Ester, Ketone and Phenol compounds were found to more likely coexist with certain endophytic fungi in the same plants. Our systematic investigation reveals that traditional medicinal plants are a rich and reliable source of novel endophytic fungi. This study was the first step towards understanding host-endophyte relationships based on the plant chemistry.

Key words: Biodiversity, endophytic fungi, host-endophyte relationships, host-preference, medicinal plants, plant chemistry,

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INTRODUCTION

Endophytic fungi frequently demonstrate single host-specificity at the plant species level but this specificity could be influenced by environmental conditions (Cohen, 2004). Some researchers use 'partial heterogeneity' or 'geographic variation' to indicate the endophytic fungal segregation impacted by environmental differences (Yahr *et al.,* 2006). Endophytes are also able to colonize multiple host species of the same plant family within the same habitat, and the distribution of some endophytes can be similar in closely related plant species. For example, most endophytic fungi belonging to *Balansieae* and originally isolated from the grass family *Poaceae* were also detected in its closely related families *Cyperaceae* and *Jun-caceae* (Marks *et al.,* 1991). On the other hand, differences in endophytic fungal assemblages have been found in different tissues of the same plant species, or even in different tissues of a single plant, which is a reflection of tissue specificity (Collado *et al.,* 2001; Frohlich *et al.,* 2001; Ganley and Newcombe, 2006). Endophytic fungi represent an important and quantifiable component of fungal biodiversity, and are known to affect plant community diversity and structure (Sanders, 2004; Gonthier *et al.,* 2006; Krings *et al,* 2007). To date, only about 80,000-100,000 fungal species have been described (Hawksworth and Rossman, 1987; Kirk *et al,,* 2001), out of a conservative estimate of 1.5 million (Hawksworth, 1991). Recent studies of endophytic fungi from tropical and temperate forests support the high estimates of species diversity (e.g., Kumar and Hyde, 2004; Santamaria and Bayman, 2005; Santamaria and Diez, 2005; Sanchez Marquez *et al,* 2007). These estimates do not include several additional sources of fungal diversity (Ganley and Newcomb , 2006).

Endophytic fungi are a group of fungi that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Hirsch and Braun, 1992). Many recent studies have revealed the ubiquity of these fungi, with an estimate of at least 1 million species of endophytic fungi residing in plants (Dreyfuss and Chapela, 1994) and even lichens (Li *et al,* 2007). A variety of relationships exist between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic (Schulz and Boyle, 2005; Arnold, 2007). Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances of potential use to modern medicine, agriculture, and industry, such as novel antibiotics, antimycotics, immuno-suppressants, and anticancer compounds (Strobel and Daisy, 2003; Mitchell *et al.,*

2008). There is great potential of finding new drugs from endophytes for treating new diseases in humans and animals (Kumar *et al.,* 2005). In addition, the studies of endophytic fungi and their relationships with host plants will shed light on the ecology and evolution of both the endophytes and their hosts: the evolution of endophyte-plant symbioses; the ecological factors that influence the direction and strength of the endophyte-host plant interaction (Saikkonen *et al,* 1998, 2004).

The relationships of endophytes with single or multiple plant hosts can be described in terms of host-specificity, host-recurrence, host selectivity, or host-preference (Zhou and Hyde, 2001; Cohen, 2006). Host-specificity is the relationship in which a fungus is restricted to a single host or a group of related species, but does not occur in other unrelated plants in the same habitat (Holliday, 1998). The frequent or predominant occurrence of an endophytic fungus on a particular host or a range of plant hosts is often defined as host-recurrence, but the fungus can also occur infrequently on other host plants in the same habitat (Zhou and Hyde, 2001). A single endophytic fungal species may form relationships with two related plant species but demonstrate a preference for one particular host, and this phenomenon is categorized as host-selectivity (Cohen, 2004, 2006). The term 'host-preference', however, is more frequently used by mycologists to indicate a common occurrence or uniqueness of the occurrence of a fungus on a particular host, and the term is also used to indicate the differences in fungal community compositions and isolation frequencies from different host plants (Suryanarayanan and Kumaresan, 2000; Bettucci *et al.,* 2004). The differences in endophyte assemblages from different hosts might be related to the chemical differences of the hosts (Paulus *et al.,* 2006).

Since natural products are likely adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes (Schulz *et al.,* 2002)., Schulz *et al.* (2002) isolated about 6500 endophytic fungi from herbaceous plants and trees over a course of 12 years, screened them for biologically active compounds, and found a correlation between biological activity and biotope, e.g. a higher proportion of the fungal endophytes, in contrast to the soil isolates, inhibited at least one of the test organisms for anti-algal and herbicidal activities. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel *et al,* 2004; Wiyakrutta *et al.,* 2004; Kumar *et al.,* 2005; Tejesvi *et al.,*

2007). The various natural products produced by endophytic fungi possess unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation for medicinal, agricultural and industrial uses (Tan and Zou, 2001; Zhang *et al.,* 2006).

The investigation study was to investigate qualitatively and quantitatively the biodiversity of endophytic fungi from 5 medicinal plant species occurring in Dhanvantri Vana, a unit of Government Ayurvedic College, Bangalore. Although there are limitations in classifying endophytes using the traditional morphological methodology, at present there are no workable alternatives (Duong *et al.,* 2006; Hyde and Soytong, 2007) since molecular phylogenetic identification is still not applicable to all fungal taxa on a large scale.

MATERIALS AND METHODS

Plant material

Five medicinal plant species such as *Caesalpinia sappan*, *Alternanthera sessil, Sapindus laurifolius, Basala alba and Acalypha indica* were collected The root hairs were collected from the five different plant. The sample was collected from Dhanvantri Vana, a unit of Government Ayurvedic College, Bangalore. The samples were collected from June 2013 to September 2014. The plants with no visible symptoms of disease were carefully selected after physical examination. The samples were kept in sterile containers and processed within few hours after sampling.

Sample collection:

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Root hair processing

The root hairs of selected 5 medicinal plants were collected and cut into 1 cm long pieces. It was disinfected with 75% alcohol for 1 minute followed by immersion in 1-5% sodium hypo chloride for 3-10 minutes. They were again immersed in 75% alcohol for 30 seconds. The root bits were then rinsed in sterile distilled water and blot dried on sterile blotting paper.

The root bits were placed on Potato Dextrose Agar (PDA) supplemented with Streptomycin. The plates were sealed with parafilm and incubated at 28ºC for 4-6 weeks.

Microscopic features:

The morphology of different endophytic fungi was studied by staining with methylene blue of the isolates from Potato Dextrose Agar (PDA) after 4-6 week incubation at 28ºC.

Organic Analysis of Fungal broth:

The crude extract of broth was studied for the presence of antibacterial compounds like phenols, aldehydes, esters, etc. the tests were performed under laboratory conditions for organic analysis. Organic compounds may be aliphatic of aromatic. They may be saturated or unsaturated. Depending upon the functional group they contain, they show different solubility and give characteristic reactions. Solubility tests were performed with dilute HCl, 1% NaOH and concentrated sulfuric acid.

Isolation of endophytic fungi

A total of 5 samples of plant roots were cut into small pieces (10 mm in length). Surface sterilization and isolation of endophytic fungi followed a modified procedure as described by Schulz *et al.* (1993), and the details of the procedure were also given in our previous study (Huang *et al.,* 2007b). Antibiotics penicillin G and streptomycin (Sigma, St. Louis, MO, USA) were added to the cultures to suppress the growth of bacteria. The pure endophytic fungal strains were photographed and preserved in the Biotechnological laboratory Indian Academy, Bangalore

RESULTS AND DISCUSSION

Biodiversity of endophytic fungi

The 12 endophytic fungal strains isolated from the 5 medicinal plants were collected from Dhanvantri Vana, a unit of Government Ayurvedic College, Bangalore. classified into 12 distinct morphospecies (Table 1). Mycelia sterilia consists of various morphological fungal types, but not forming true spores. This group of fungi is considerably prevalent in endophyte studies (Lacap *et al.,* 2003). In the 12 frequently encountered endophytic fungal groups, Branched septet mycelium, conidiophores erect, globosa vesicle with uniseriate sterigmata which produces chain of conidia by basipetal succession.

mycelia sterilia had the highest relative frequency (10.16%) in the 5 medicinal plants. *Colletotrichum* which are frequently identified as endophytes (Photitia *et al.,* 2005;

Devarajan and Suryanarayanan, 2006) was the second most frequent endophytic group, followed by *Aspergillus spp, Bispora spp., Geotrichum spp., Trichoderma spp. Cladosporium spp. Aspergillus spp. Gliocladium spp., Trichoderma spp. Fusarium spp. Trichosporoniodes spp Trichoderma spp*

with relative frequencies (Fig. 1). These were the dominant genera or order of endophytic fungi found in this study, similar to the findings reported previously for many tropical endophytic fungi (Corrado and Rodrigues, 2004; Krohn *et al.,* 2007). The 18 infrequent endophytic fungal species or genera included *Aureobasidium pullulans, Botryo-sphaeria, Chaetomella, Chaetomium, Clado-sporium,* Coelomycetes, *Drechslera-Yike, Ellisembia, Ephelis Flagellospora, Helmin-thosporium, Pestalotiopsis, Physalospora, Pyrenochaeta, Pyriclilariopsis, Rhizosphaera, Spiropes,* and *Verticillium.* Among them, *Spiropes, Ellisembia,* and *Helminthosporium* are reported here for the first time as endophytic fungi.

The endophytic fungi are very unique in their properties, which infect only the plant parts like root. They differ from the rhizosphere soil in their qualitative microflora niche. They can be considered obligatory parasites infecting only the plants. Very few can be facultative in existence surviving in the rhizosphere soil and also as an endoparasite in plants. The isolated fungi were maintained as a stock on PDA slants. The fungi isolated in endophytic are as follow:

Sr. No.	Plant name	Medicinal Properties	Endophytic Fungi
1.	*Caesalpinia sappan*	Diarrhoea	*Colletotrichum spp.* *Aspergillus spp.* *Bispora spp.* *Geotrichum spp.*
2.	*Alternanthera sessilis*	Skin rashes, Liver disorders	*Trichoderma spp.* *Cladosporium spp.* *Aspergillus spp.*
3.	*Sapindus lourifolius*	Diarrhoea, White patches on skin	*Gliocladium spp.* *Trichoderma spp.*
4.	*Basala alba*	Mouth Ulcer, body energy	*Fusarium spp.* *Trichosporoniodes spp*
5.	*Acalypha indica*	Skin disease , cough	*Trichoderma spp.*

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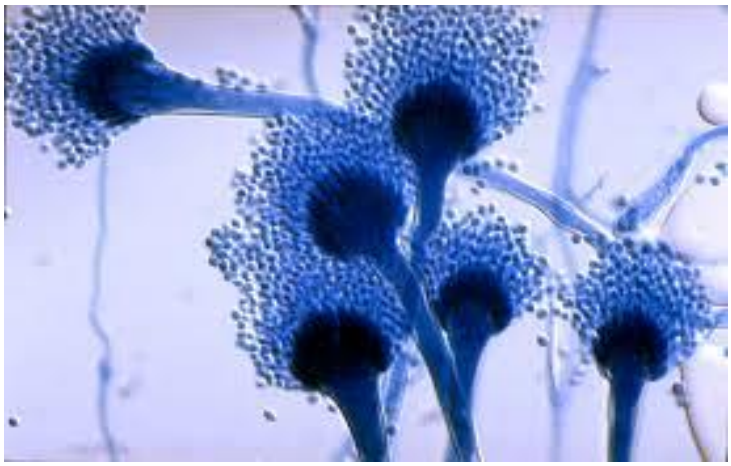
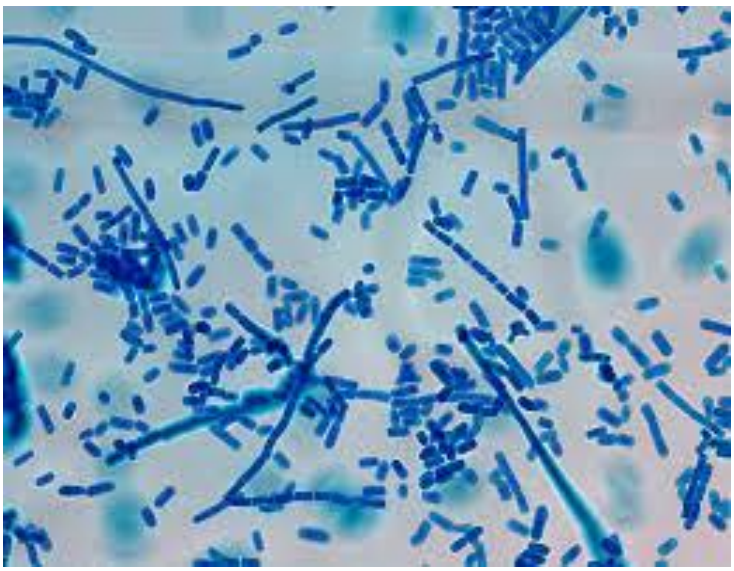


Aspergillus *spp.*

Bispora



Geotrichum

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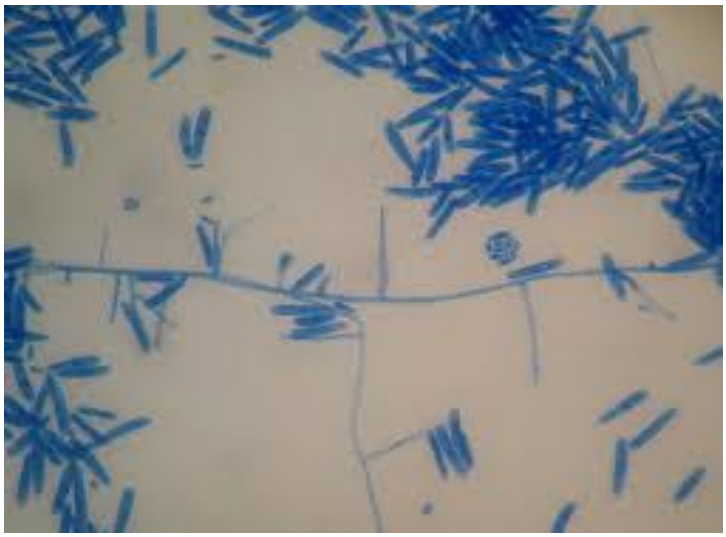
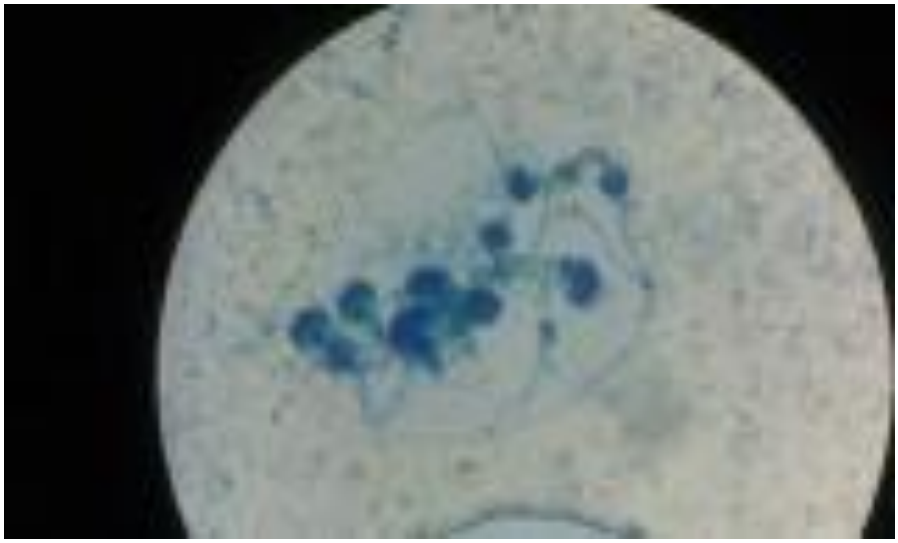


Fusarium

Helminthosporium



Gliocladium spp

Morphological characteristic:

The morphology of isolated fungi were studied under microscope after staining with methylene blue stain.

The characteristics of fungal isolates are tabulated as below:

Fungal Isolate	Characteristic features
Aspergillus spp.	Branched septet mycelium, conidiophores erect, globosa vesicle with uniseriate sterigmata which produces chain of conidia by basipetal succession.
Bispora spp.	Mycelium dark; conidiophores dark, short simple, sparingly branched; conidia dark, oblong to ellipsoid, 2-celled or less with thick black septa, saprophytic.
Colletotrichum spp.	Acervuli is disc shaped, waxy. Conidiophores are simple, elongated, conidia hyaline, ovoid and parasitic.
Cladosporium spp.	Septate mycelium, conidiophores tall, dark and clustered. Conidia (blastospores) dark, 1-2 celled, ovoid to cylindrical, lemon shaped. Saprophytic or parasitic.
Fusarium spp.	Mycelium extensive with slender conidiophores, whorl of phialids grouped as sporodochium, micro conidia 1-celled ovoid, macor conidia is 2-3 celled, canoe or sickle shaped.
Gliocladium spp.	Conidiophores like penicillate brushes, conidia in mucilaginous droplets. Shows a stage Verticillium. Saprophytic in soil.
Geotrichum spp.	Mycelium white, septate; conidiophores absent; conidia hyaline, single celled, short cylindrical with truncate ends, mostly saprophytic.
Trichoderma spp.	Conidiophores branched, non verticillate, conidia in terminal clusters. Saprophytic in soil and wood.
Trichosporoniodes spp.	True and pseudo mycelium, conidiophores simple with swollen globose apex bearing conidia (botryo blastospores) on sterigmata, conidia hyaline single celled yeast like.

Previous studies reported distinct endophyte community compositions in different host plants suggesting host preferences (Cannon and Simmons, 2002; Cohen, 2006). This study also found the significant differences in both presence /absence and relative abundance of fungal endophytes in the medicinal plants occurring in Bangalore. The colonization rate and the isolation rate of endophytic fungi from these plants varied greatly. Some medicinal

plants harbored more endophytic fungi than others. The number of fungal taxa colonizing these hosts ranged from three to 15, and many of the fungal taxa had different isolate frequent-cies in different hosts. Some of the common endophytes not only existed in more plant hosts but also had higher relative frequencies within each of the hosts.-In contrast; some other endophytic fungi were detected in only one given plant host. These descriptions of host-preference were consistent with previous reports (Arnold *et al.,* 2001; Bettucci *et al.,* 2004).

The endophytic fungi in these 5 medicinal plants exhibited tissue-specificity. Some fungal endophytes were more likely found in the roots while others in the stems. Some infrequent fungal endophytes were found in only one type of tissue (leaf /stem/root). The difference in endophyte assemblages from various tissues indicated that some fungal endophytes have an affinity for different tissue types and this might be a reflection of their capacity for utilizing or surviving within a specific substrate (different tissue texture and chemistry) (Rodrigues, 1994; Photita *et al.,* 2001). Many previous reports also discovered tissue-specificity in endophytic fungi (e.g., Taylor *et al.,* 2001; Ganley and Newcombe, 2006).

Spatial variability has not been thoroughly explored for endophytic fungi and may be difficult to discern because stratum, substrate, or host preferences confound spatial patterns (Arnold *et al,* 2000). In this study, among-site differences were found in endophytic composition and abundance, but this spatial variation may contain the component of host-preferences as the collection sites also differ in plant composition. Some sites harbored more fungal endophytes than the others, and some endophytes were found in only one location. Spatial heterogeneity in the distribution of endophytes was also reported in previous studies (Arnold *et al.,* 2001; Gallery *et al.,* 2007). Such spatial heterogeneity may be partly due to differences in environmental conditions, including humidity, temperature, rainfall and potential inoculum sources (Photita *et al.,* 2001; Santamaria and Dayman, 2005).

The host plants in our study possessed different phenolic compounds (e.g., Aliphatic compound phenolic acids, Aromatic compound , Acid, Amine, Aldehyde, Ester, Ketone

and these different hosts were colonized by various different endophytic fungi. Some correlation apparently exists between the endophytic fungal assemblages and the host chemistry. Unfortunately, it was impossible to identify or quantify neither all the chemical compounds nor all the endophytic fungi present in the plant hosts. In this study, only the

total contents of phenolics and 5 hosts were investigated and their major phenolic compounds and fungal endophytes identified. However, the results suggested that the total contents of phenolics and flavonoids of the host plants influence both the quantity of endophytic fungal taxa and the number of endophytic isolates. Moderate TPC and TFC appear to favor the growth of endophytic fungi. The plants with too low or too high TPC and TFC were colonized with fewer endophytic fungi. Based on the established matrix, some phenolic compounds were found to more likely coexist with some endophytic fungal taxa, such as chlorogenic acid with mycelia sterilia, *Colletotrichum,* and *Phomopsis,* rutin with *Colletotrichum,* and *Aspergillus spp, Bispora spp.,*Although some endophytic fungi may prefer the hosts with specific compounds, the presence of a given compound could not guarantee the presence of a given endophyte. The quantity of the specific compounds could also play a role in fungal colonization. Furthermore, the colonization of endophytes can induce the plants to produce certain compounds, and some of the special compounds with small quantity might be actually produced by the endophytes within the host plants. There exist other hypotheses that microbial symbionts could affect plant nutrition, defensive chemistry, and biodiversity (Rudgers *et al,* 2007). The host-endophyte relationship is complex and involves many factors, but studying the presence-absence of a given endophyte in the presence of a given compound of the host plant is an important first step toward our understanding of this intricate relationship. Further studies are needed to reveal the interaction between the host plant and its endophytes.

In conclusion, this study investigated endophytic fungal diversity and host-endophyte relationship based on traditional metholody. The 12 endophytic fungal isolates from 5 medicinal plants were identified and classified. *Colletotrichum spp, Aspergillus spp, Bispora spp., Geotrichum spp., Trichoderma spp. Cladosporium spp. Aspergillus spp. Gliocladium spp., Trichoderma spp. Fusarium spp. Trichosporoniodes spp Trichoderma spp* were the dominant fungal taxa. The evidence for host-preference, tissue-specificity, and spatial heterogeneity was found in the endophyte distribution based on fungal community compositions and isolation frequencies. Certain correlations exist between the endophytic fungal assemblages and the host plant chemistry.

The Studying. endophytes as we have done here is a method-dependent process (Quo *et al.,* 2001) and thus the fungi isolated are dependent on our methodology. We were also unable

to identify the mycelia sterilia. Future investigation should be use molecular sequence data to identify mycelia sterilia (e.g. Wang *et al.,* 2005; Promputtha *et al.,* 2005; Sanchez Marquez *et al.,* 2007). Total fungal communities should be detected by extracting the entire host DNA (for example from roots) with various methods to sequence individual taxa. Potentially

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