EFFECT OF DUAL INOCULATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON DIFFERENT NON-LEGUMINOUS PLANTS UNDER POT CONDITION

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ABSTRACT

Purpose: *Purpose of the present study is to check the potency of the strains Bacillus thuringiensis A5-BRSC and Bacillus megaterium ATCC 9885 for promoting growth of different plants without hazardous chemical fertilizers.*
Methods: *Four non-leguminous seasonal plants like Amaranthus; Ocimum, Abelmoschus and Capsicum are used to study combined growth promoting effects of two bacteria viz. Bacillus thuringiensis A5-BRSC and Bacillus megaterium ATCC 9885 under pot condition. Different morphological parameters like shoot height, leaf area, fresh weight and dry weights of the plants; as well as biochemical parameters like leaf chlorophyll content, catalase and peroxidae enzyme activities of both culture treated and untreated plants were investigated.*
Results: *All the biofertilizer treated plants showed remarkably higher results in all parameters, except chlorophyll content, than control plants.*
Conclusion: *The strains proved as potential strains for plant growth promotion in pot study and hence may be used as biofertizer under field condition for sustainable agriculture.*

Key words: *Bacillus thuringiensis, Bacillus megaterium, dual inoculation, pot culture*

INTRODUCTION

Plant growth promoting effects of Plant Growth Promoting Rhizobacteria (PGPR) in pot as well as in fields were exhaustively studied for last three decades. Key objective of such kind of study is to exhibit growth promoting effects of PGPR strains either in pot or in field condition. Such strains are widely used as biofertilizer for sustainable agriculture. Jeon et al. [1] reported that particular strains of *Pseudomonas fluorescens, Bacillus megaterium and Azotobacter vinelandii* accelerated the rate of growth of different wild plants in pots under greenhouse condition. Wu et al. [2] reported the combined effect of nitrogen fixing, phosphate and potassium solubilizing fungi in the growth of maize under greenhouse condition. Effect of *Pseudomonas putida* and *Trichoderma attoviride* in growth stimulation of tomato plants under greenhouse state was reported by Gravel et al. [3]. It was reported that inoculation of those microorganisms not only stimulates in the increase of root length and shoot length, but accelerates in the secretion of Indole Acetic Acid (IAA) also. Falik et al. [4] demonstrated that PGPR inoculation in sorghum plants showed improved water potential under field condition. Combined effect of different PGPR belong to the genus *Azospirillum, Azotobacter, Mesorhizobium, Pseudomonas* in leguminous plant chickpea was also reported by Rokhzadi et al. [5] under field condition. Their study showed that dry matter accumulation in different plant organs was significantly affected by inoculation with PGPR in the flowering stage. Inoculation of PGPR also affected on grain yield and biomass concentration. Grain yield was almost doubled in inoculated plants in comparison to control plants. Total protein content of the grains collected from the inoculated plants also showed a sharp increase than control. Datta et al. [6] also reported the same in PGP strains of *Bacillus* and *Streptomyces*. They reported that combined effect of those microorganisms enhanced shoot height, canopy width, total number of fruits, fruit weight, and fruit length in chili (*capsicum annum*) plants under field condition.

It was reported that length of lateral roots and root diameter was increased in maize by inoculation with IAA producing PGPR *Azospirillum* [7]. Levanony and Bashan [8] showed that inocution of *A. brasilense* enhanced cell division in the root tips of wheat plants. Okon and his

associates [9, 10] reported that inoculation of *Azospirillum* in cereal crops and tomato plants respectively increased root hair formation. Mia et al. [11] also reported the same result in tissue cultured banana plants during pot culture study. They observed that introduction PGPR in pot not only increased initial root hair formation, but also increased plant height, leaf area, total leaf chlorophyll content and total dry matter accumulation. The effect of PGPR *P. putida, P. fluorescens, A. lipoferum* and *A. brasilense* on germination, seedling growth and maize yield was also reported in both pot cultures as well as under field conditions [12]. Their results showed that bacterial inoculants did not affect on the leaf and shoot weight under pot condition, but those parameters were significantly increased in field condition. They also concluded that bacterial inoculants significantly increased seed germination rate. The rate of seed germination was calculated by Vigor Index [VI = (mean root length + mean shoot length) x % of seed germination]. *A. brasilense* was reported to be the strain with highest VI, followed by *P. putida* (strain R-168). Cyanobacteria like *Anabaena, Scytonema, Oscillatoria* and *Lyugbya* was also reported to act as good inoculants in pot culture [13]. Inoculation of cyanobacterial culture induced an appreciable increase of chlorophyll-a, chlorophyll-b and total chlorophyll content in leaves. It also increased root and shoot length as well as enhances plant catalase and peroxidase activity significantly. Mia et al. also reported inoculation of PGPR in pot increased root length, shoot length, biomass and nitrogen accumulation in tissue cultured banana plants [11].

Survival of PGPR microbes under pot condition were measured by various ways. Total microbial activity in soil may be measured by soil Dehydrogenase activity or Electron Transfer System (ETS) activity assay [14] where Triphenyl Tetrazolium Chloride (TTC) in converted to triphenyl formazan (TPF) by dehydrogenase enzyme released by the aerobic microflora of the soil. The amount of TPF is measured spectrophotometrically. Carbon di-oxide evolution method was also reported as a method of choice for determining the activity of aerobic microorganisms in soil, in which the amount of oxidized carbon (i.e., CO_2) produced due to respiration of aerobic microflora of pot soil, was measured [15]. High rate of CO_2 production by soil microflora indicates high metabolic activity of the microorganisms.

MATERIALS AND METHODS

Preparation of pots & Cultivation of seeds: Surface loamy soil from a bare ground area of local field ($22^0$44'N $88^0$25'E) was collected and sieved to discard small pieces of bricks and stones. About 5 kg of loamy soil (pH- 7.2; moisture content: 36±2%; Electronic conductivity: 0.3 ds/m; Organic carbon: 0.52±0.3%) was taken in each pot. Seeds of *Amaranthus viridis*; *Ocimum tenuiflorum, Abelmoschus esculentus* and *Capsicum annuum* were eventually distributed on the surface of each of the four sets of pots. 20 pots were selected for each of the four different sets of plants. All the experiments were done in spring season (month of March-April)

Preparation & application of inoculum: Phosphate solubilizing and phytohormone producing strain of *Bacillus thuringiensis* A5-BRSC was screened from the agricultural soil and it was identified by 16s r DNA analysis [16]. The phosphate solubilizing strain *Bacillus megaterium* ATCC 9885 was purchased from Hi-media (India). Each culture of A5-BRSC and *B. megaterium* with a cell density of $2x10^6$ CFU/ml was mixed with charcoal powder (1:1 ratio w/v) and applied to the soil of the pot after 7 days of sowing of seeds. One set of un-inoculated pots for each of the three plants served as control. All inoculated and un-inoculated control pots were maintained in a sunny area and water was sprayed regularly.

Measurement of plant heights: Growth of the plants (shoot height), leaf area, numbers of leaves, flowering time and length of fruits were monitored for 75 days in every 15 days of interval. Wet weight and dry weight of the plants was taken after 75 days.

Estimation of microbial activity in pot: Total activity of aerobic microorganisms in both test and control pots were determined by carbon di-oxide evolution method and soil dehydrogenase assay. In every 15 days

interval 60 g of soil from each of the pots were withdrawn and divided into two parts. One part of the soil was taken for carbon di-oxide evolution assay and another part was taken for soil dehydrogenase assay. 50 g of moistened soil from each pot was collected in 1 l conical flask and a test tube with 15 ml of 0.5 N NaOH was kept inside the flask. The NaOH solution was previously titrated with primary standard 0.5 N oxalic acid solution. The flask was incubated for 24 hours at 30°C by sealing it with parafilm. An empty conical flask (without soil) was treated in the same manner as 'negative control'. Next day the residual NaOH was titrated with standard 0.5 N HCl in both negative control and soiled pots. The amount of carbon evolved was calculated by using the formula: (M.W. of carbon / M.W. of CO_2) x volume of HCl consumed.

For soil dehydrogenase assay 6 g of air-dried soil was thoroughly mixed with 0.6 g of $CaCO_3$ and the mixture was taken in sterile culture tube. 2.5 ml of sterile water and 1 ml of 3 % aqueous Triphenyl Tetrazolium Chloride (TTC), the substrate, was added to the tube. One tube without soil was treated in the same manner acted as control. All the tubes were incubated for 24 hours at 30°C. After 24 hours 10 ml of 100 % methanol was added to each tube and the content was filtered through Wattman filter paper no.5 and the filtrate was collected. The absorbance of the filtrate was noted in spectrophotometer at 485 nm. The amount of product formed was estimated from the standard curve of the product Tri Phenyl Formazan (TPF). Activity of 1 ml of the enzyme was expressed as µmoles of product formed /min under the specified assay condition.

Determination of chlorophyll content: Chlorophyll content of leaves was determined by following the method of Dere et al., 1998 [17] with slight modifications. 1 g of youngest fully expanded leaf from each plant was put in 50 ml of 96% methanol. The mixture was homogenized at 2000 rpm for 1 minute. Homogenate was filtered through two-layered cheese cloth filter. The filtrate was centrifuged at 2500 x g for 10 minutes. Absorbance was taken to determine the amount of chlorophyll a, chlorophyll b and total carotene present in leaf extract.

Catalase and Peroxidase enzyme activity of leaves: Catalase and peroxidase activity of the leaves were estimated by following the protocol described by Chance and Maehly [18] with slight modification. 0.5 g of leaf samples were crushed in pre-chilled mortar-pestle and homogenized with 10 ml of 0.1 M cold phosphate buffer, pH: 7.0. The homogenate was centrifuged at 17000 x g for 10 minutes at 4°C and diluted 5 times with 0.1M phosphate buffer, pH: 7.0. This diluted sample was taken as enzyme source for biochemical studies. To determine catalase activity 1 ml of this leaf extract was mixed with 2 ml of 0.1 M phosphate buffer, pH: 7.0 and 2 ml of 100 µmoles of H_2O_2 at 30 °C for 1 minute. The reaction was terminated by adding 2 ml of 6N H_2SO_4. Residual H_2O_2 was titrated against 0.01 N $KMNO_4$. A control set was treated in the same manner where the enzymatic reaction was stopped at zero time. One unit of catalase activity was expressed as the amount of enzyme that breaks down 1 µmole of H_2O_2 /min under the specified assay condition. For peroxidase assay 1 ml of 10-times diluted leaf extract was mixed with 1 ml 0.1 M phosphate buffer, pH: 7.0, 1 ml of 50 µmoles of pyrogallol and 2 ml of 100 µmoles of H_2O_2. Reaction mixture was incubated at 30°C for 5 minutes and the reaction was stopped by adding 2 ml of 6N H_2SO_4. The colour of purpurogallin formed was measured by taking absorbance at 420 nm. Activity of 1 ml of the enzyme was expressed as µmoles of product formed /min under the specified assay condition.

RESULT

Dual inoculation of bacterial culture in each pots, containing the seeds of *Amaranthus viridis*, *Capsicum annuum* (Chilli), *Abelmoschus esculentus* (Vindi) and *Ocimum tenuiflorum* (Tulsi) showed that germinated seeds remarkably grow higher than that of un-inoculated pot in 45 days (Figure 1). Induction of growth of experimental plants in presence of the inoculum in pot is clearly evident from the data shown in Figure 1. Growth rate of vindi plant was fastest among the test plant sets. Inflorescence of tulsi and *Amaranthus*

occurred after 32 and 21 days of inoculation respectively. Fluorination and fruit formation in chili and vindi plants were observed after 24 & 38 days and 22 & 30 days of inoculation respectively. Microbial activities in biofertilizer inoculated pots of *Abelmoschus* plants showed remarkably higher values than control pots (Figure 2). All other pots of three test plants also showed higher rate of soil dehydrogenase assay and carbon di oxide evolution than control pots (data not shown). Moreover, experimental plants exhibited significantly higher catalase and peroxidase enzyme activities than control plants; although biofertilizer inoculation did not increased leaf chlorophyll content in appreciable amount (Table 1, 2, 3 & 4).Significant differences was analyzed by one-way ANOVA at 0.05 levels.

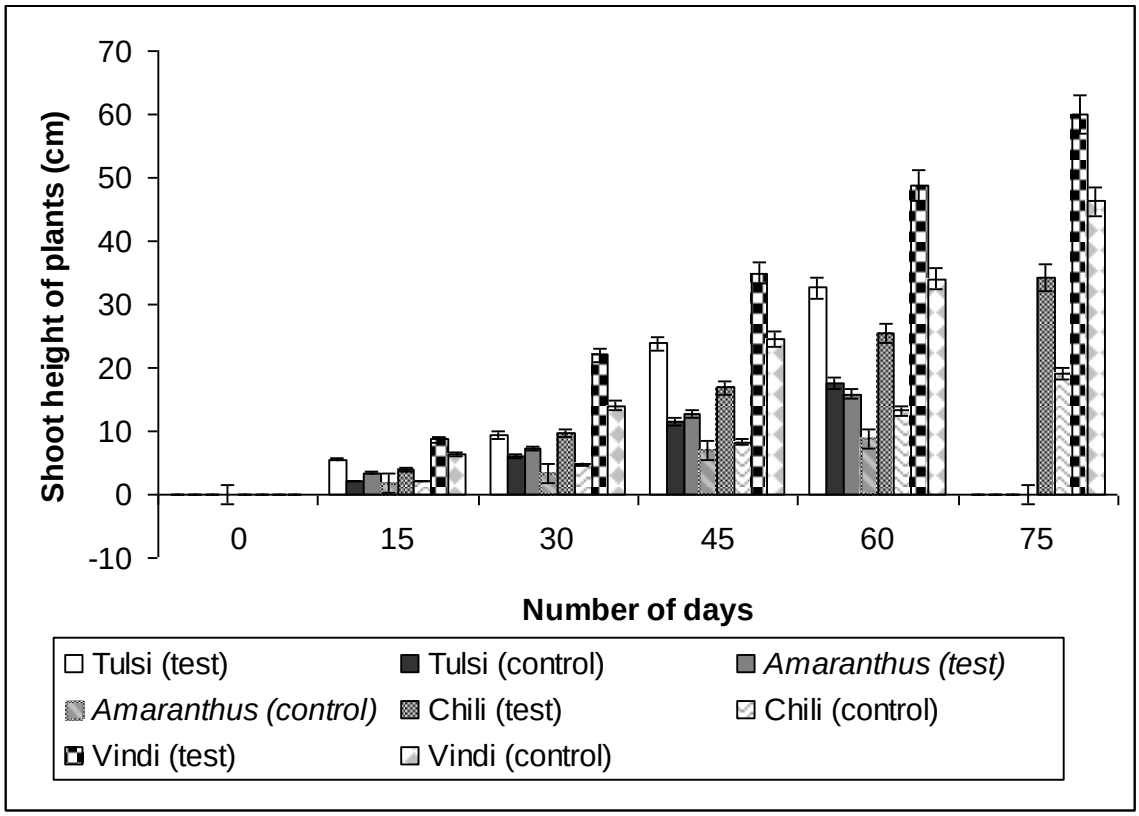

Figure 1: Shoot height record of different experimental plants in pot culture study
(It should be noted that there was no significant increase of shoot height took place in case of Tulsi and *Amaranthus* at 75 days)

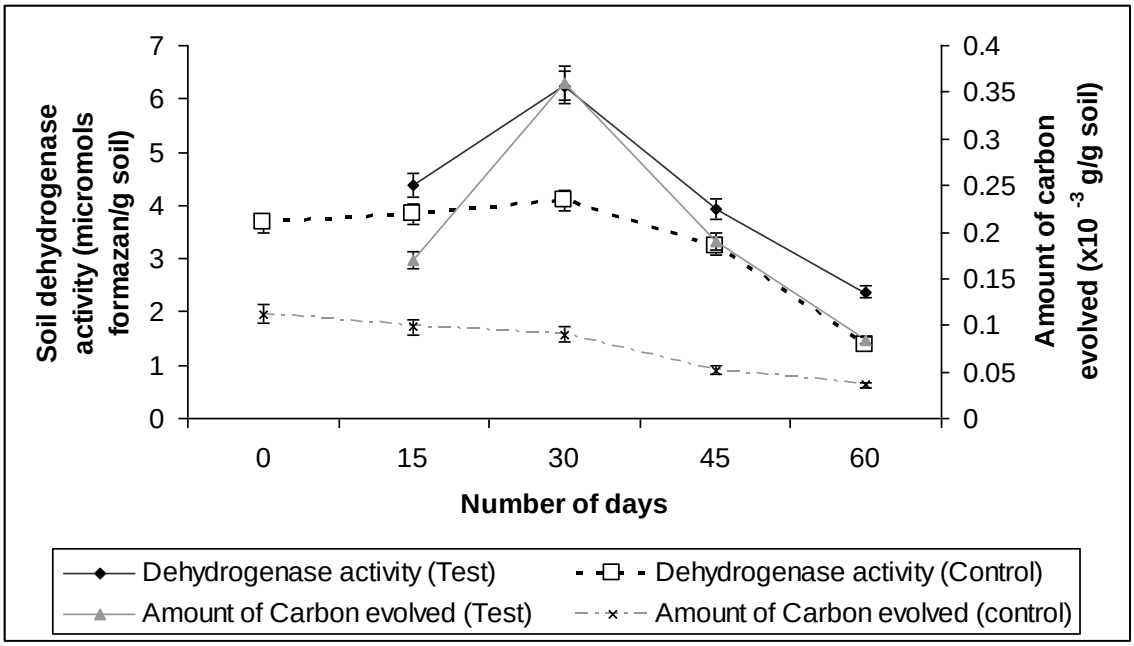

Figure 2: Measurement of microbial activities in inoculated pots of *Abelmoschus esculentus*, compared to control pots by soil dehydrogenase assay and CO_2 evolution method

Table 1: Comparative biochemical and morphological analysis of Tulsi (*Ocimum tenuiflorum)* plant in both biofertilizer inoculated and un-inoculated pots

Treatments	**Shoot length (cm)**	**Leaf area (cm^2)**	**Leaf chlorophyll content (mg/cm^2)**	**Fresh weight of plant (g)**	**Dry weight of plant (g)**
B. thuringiensis A5-BRSC & *B. megaterium* ATCC 9885	33.2± 0.2	37.0 ± 0.22	Chl.a: 0.61 ± 0.55* Chl. b: 0.43 ± 0.15 Total Chl: 1.09 ± 0.3	21.88 ± 0.5	5.27 ± 0.11
Control	17.6± 0.42	26.7 ± 0.15	Chl.a: 0.54 ± 0.18* Chl. b: 0.28 ± 0.11 Total Chl: 0.84 ± 0.23	15.9 ± 0.22	2.89 ± 0.25

(Note: Abbreviation: Chl. = Chlorophyll; All data shown in Mean ± standard error; *Not significant in 0.05 level of ANOVA)

Table 2: Comparative biochemical and morphological analysis of *Abelmoschus esculentus* plant in both biofertilizer inoculated and un-inoculated pots

Treatments:	***B. thuringiensis* (A5-BRSC) & *B. megaterium* as biofertilizer in pots**	**Control pots**
Leaf chlorophyll content (mg/cm^2)	Chl.a: 1.097 ± 0.38* Chl. b: 0.582 ± 0.76 Total Chl: 1.695± 1.01*	Chl.a: 1.115± 0.35* Chl. b: 0.495± 0.55 Total Chl: 1.712± 0.88*
Peroxidase activity (U/g leaf)	41 ± 1.8	27 ± 1.7
Catalase activity (U/g leaf)	176 ± 0.89	110 ± 1.2
Fresh weight of plant (g)	Root: 19.34 ± 0.5; Leaf: 42.25 ± 0.6; Stem: 58.02 ± 0.42 Total: **121.01 ± 1.2**	Root: 13.6 ± 0.22; Leaf: 27.3 ± 0.62; Stem: 42.19 ± 0.5, Total:**83.28 ± 1.4**
Dry weight of plant (g)	23.02 ± 0.14	14.8 ± 0.11

(Note: Abbreviation: Chl. = Chlorophyll; All data shown in Mean ± standard error; *Not significant in 0.05 level of ANOVA)

Table 3: Comparative biochemical and morphological analysis of 60 days old *Capsicum annuum* plant in both biofertilizer inoculated and un-inoculated pots

Parameters measured	***B. thuringiensis* A5-BRSC & *B. megaterium* as biofertilizer in pots**	**Control pots**
Leaf chlorophyll content (mg/cm^2)	Chl.a: 0.962 ± 0.22* Chl. b: 0.425 ± 0.13* Total Chl: 1.358± 0.78	Chl.a: 0.955± 0.3* Chl. b: 0.412± 0.18* Total Chl: 1.182± 0.52
Peroxidase activity (U/g leaf)	27.6 ± 0.92	15.3 ± 0.85
Catalase activity (U/g leaf)	130 ± 0.8	90 ± 0.25
Fresh weight of plant (g)	Root: 7.68 ± 0.2; Leaf: 17.28 ± 0.32; Stem: 29.87 ± 0.55 Total: **54.97 ± 1.7**	Root: 4.63 ± 0.3; Leaf: 7.19 ± 0.5; Stem: 21.99 ± 0.45; Total:**33.98 ± 1.2**
Total Dry weight of plant (g)	13.0 ± 1.2	9.47 ± 0.8

(Note: Abbreviation: Chl. = Chlorophyll; All data shown in Mean ± standard error, *Not significant in 0.05 level of ANOVA)

Table 4: Comparative biochemical and morphological analysis of 60 days old *Amaranthus* plant in both biofertilizer inoculated and un-inoculated pots

Parameters measured	***B. thuringiensis* A5-BRSC & *B. megaterium* as biofertilizer in pots**	**Control pots**
Leaf chlorophyll content (mg/cm^2)	Chl.a: 0.62 ± 0.12* Chl. b: 0.25 ± 0.15* Total Chl: 1.01± 0.55*	Chl.a: 0.52± 0.11* Chl. b: 0.21± 0.18* Total Chl: 0.982± 0.25*
Peroxidase activity (U/g leaf)	15.8 ± 0.2	12.3 ± 0.5
Catalase activity (U/g leaf)	110 ± 0.3	95 ± 0.2
Fresh weight of plant (g)	46.7 ± 1.1	31.98 ± 1.9
Total Dry weight of plant (g)	21.0 ± 0.9	18.07 ± 0.5

(Note: Abbreviation: Chl. = Chlorophyll; All data shown in Mean ± standard error; *Not significant in 0.05 level of ANOVA)

DISCUSSION

The result represented in this work reflected that the strains are able to promote the growth of different vegetable plants in pot condition. Recent work on application of PGPR in crop development implies the use of such PGPR strains that work well in both pot and field studies [5, 6, 12]. All the plants used in the present study are non-legumious and hence none of these plants can act as natural habitat for nodulating nitrogen fixing bacteria. Therefore the effect of only rhizobacteria associated with the soil and biofertilizer can interact with the plant. Moreover, all the plants chosen for this study are either herb or shrub and annual according to their life span so that they can be easily handled in pots. Inoculation of *Bacillus thuringiensis* (A5-BRSC) in pots increased the root length, shoot height and diameter, leaf area, fruit weight, fresh weight and dry weight of plants. Total amount of catalase and peroxidase activities of leaves are significantly increased in test plant during pot study, although leaf chlorophyll content is not significantly higher in test plants, in comparison to control. Same pattern of result was also described by Mia et al. [11]. Their study with tissue cultured banana plant in pot condition revealed that introduction PGPR in pot not only increased initial root hair formation, but also increased plant height, leaf area, total leaf chlorophyll content and total dry matter accumulation. Jeon et al [1] reported that combined effect of *Pseudomonas* strains showed initial low rise of dehydrogenase activities in soil, where as our present study showed a rapid peak of soil dehydrogease activities in culture treated pots (Figure 2) within 10 days of inoculation and maintained an appreciable amount of dehydrogenase content up to 60 days. The result clearly indicated that microbial activities in pots for a long period of time directly involved to the promotion of plant growth. Growth promoting effects of PGPR in chilli plants were also reported by Datta et al [6] under both field and pot condition. Inoculation of consortia in pots showed significant higher plant height, fruit weight, total number of fruits per plant and other morphological parameters which were quite consistent with the present study. Similar pattern of results in different non-leguminous plants were also indicated by Basan et al.; Mia et al. and other researchers [8, 11].

CONCLUSION

Combined effect of the strains *B. thuringiensis* A5-BRSC and *B. megaterium* ATCC 9885 used in the present study was proved to accelerate the growth rate of plants and therefore, it has tremendous potential to be used as biofertilizer in field condition for better crop production in future.

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