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Herbal Drug Quality and Phytochemical Composition of *Hypericum perforatum* L. Affected by Ash Yellows Phytoplasma Infection

RENATO BRUNI,*,† FEDERICA PELLATI,‡ MARIA GRAZIA BELLARDI,§ STEFANIA BENVENUTI,‡ SAMANTA PALTRINIERI,§ ASSUNTA BERTACCINI,§ AND ALBERTO BIANCHI†

Dipartimento di Biologia Evolutiva e Funzionale, Sez. Biologia Vegetale e Orto Botanico, Università degli Studi di Parma, Parco Area delle Scienze 11A, 43100 Parma, Italy, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy, and Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA), Patologia Vegetale, Università degli Studi di Bologna, Viale Fanin 42, 40126 Bologna, Italy

Qualitative/quantitative phytochemical variations were observed in dried flowering tops of cultivated *Hypericum perforatum* L. cv. Zorzi infected by phytoplasmas of the "ash yellows" class, identified by direct and nested polymerase chain reaction (PCR); this is the first report of ribosomial group 16SrVII phytoplasmas in St. John's Wort. Methanolic extracts of healthy and infected plants were separated by reversed phase high-performance liquid chromatography to quantify naphthodianthrones and flavonoids, while essential oils were analyzed by means of gas chromatography (GC)−GC/MS. The affected plants exhibited decreased amounts of rutin (1.96 ± 0.23 vs 4.96 ± 0.02 mg/g), hyperoside (2.38 ± 0.21 vs 3.04 ± 0.05 mg/g), isoquercitrin (1.47 ± 0.04 vs 3.50 ± 0.08 mg/g), amentoflavone (0.12 ± 0.01 vs 0.39 ± 0.02 mg/g), and pseudohypericin (1.41 ± 0.23 vs 2.29 ± 0.07 mg/g), whereas the chlorogenic acid content was doubled (1.56 ± 0.11 vs 0.77 ± 0.02 mg/g). Hypericin, quercitrin, and quercetin contents were not severely affected. The essential oil yield was drastically reduced in infected material (0.11 vs 0.75% in healthy material) and revealed an increased abundance of sesquiterpenes (β-caryophyllene, δ-elemene, and germacrene D, in particular) and a matching decrease in monoterpene hydrocarbons and aliphatics. The consequences that the phytopathological condition of cultivated *H. perforatum* plants has on the commercial quality, market value, and therapeutic efficacy are outlined.

KEYWORDS: Phytoplasma disease; *Hypericum perforatum*; cultivation; hypericins; flavonoids; essential oil

INTRODUCTION

Over the last few decades, induced by a prosperous herbal market, trends in herbal cropping have led to increased cultivation of medicinal plants, herbs, and spices. With these new particular crops, however, unique diseases and pest problems are emerging. Some of these were previously rare or unknown in the wild and have been promoted by the use of agricultural systems (*1*). In terms of production, it is well-known that plant pathologies can cause considerable losses in gross medicinal plant yield. Nevertheless, raw harvesting yield is not the sole key factor in determining the quality (and hence the market value) of herbs intended for the phytotherapeutic market. The quality and therapeutic value of the products are, in fact, also determined by the abundance of specific secondary metabolites reputedly responsible for the pharmacological activity of medicinal plants. As a direct consequence of its raison d'être, it is generally known that secondary metabolism in plants can be altered in response to biotic and abiotic factors, including exposure of individual plants to pathologies. However, only in few cases has the extent of such changes been investigated.

Phytoplasmas or mollicutes, once called mycoplasma-like pathogens, are wall-less, noncultivable intracellular prokariotes that proliferate in the phloem of affected plants and are responsible for various diseases affecting several hundred plant species (*2*). In most cases, phytoplasma infections are an overriding concern in agricultural systems, as their spread can lead to serious losses in many cash crops and cultivations (i.e., cocoa, apple, coconut, grapevine, and potato) (*3*).

* To whom correspondence should be addressed. Fax: 0039 0521 905403. E-mail: bruni@biol.unipr.it.
† Università degli Studi di Parma.
‡ Università degli Studi di Modena e Reggio Emilia.
§ Università degli Studi di Bologna.

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Until such molecular tools as polymerase chain reaction (PCR) became readily available, research into these pathogens was fairly limited (*2*), and because of difficulties in taxonomic identification, the correlation was seldom made between their presence and the effects on the host plant secondary metabolism. Recent developments have dramatically improved our knowledge of the physiochemical relationships between phytoplasma and host plants; yet, very little data are available regarding the effect that phytoplasma infection has on the quality of cultivated medicinal plants (*2*, *3*). This is primarily because the epidemiological study of mollicutes spreading inside such crops has only become routine in recent years (*4*). Diverse phytoplasma infections have recently been found infecting various cultivated medicinal plants: *Galega officinalis* L., *Digitalis lutea* L., *Hyssopus officinalis* L., *Parietaria officinalis* L., *Tagetes patula* L., *Spartium junceum* L., and *Vinca rosea* L. (*2*, *4*). Furthermore, during the spring and summer of 2002, plants grown in an experimental field of *Hypericum perforatum* were found to be infected with phytoplasmas of the ash yellows group; thus, joint diagnostic−phytochemical studies were performed.

H. perforatum L. (Hypericaceae = Guttiferae) is presently considered one of the few economic plants that has completed the transition from noxious weed to wild collected resource and then to successfully cultivated crop. Because of its well-established market position, its popularity, and its efficacy, *H. perforatum* is one of the best-selling herbs of the past decade (*5*). The drug consists of the dried flowering tops or aerial parts of the plant. Its major constituents include naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin and adhyperforin), flavonoids (hyperoside, quercitrin, isoquercitrin, rutin, and quercetin), biflavones (amentoflavone and biapigenin), phenylpropanes (chlorogenic acid), and an essential oil rich in sesquiterpenes (*6*). In phytomedicine, the whole extract and some defined phytochemicals are responsible for a plethora of pharmacological properties, ranging from wound healing (*7*) and antiseptics (*8*) to antiviral (*9*), antiinflammatory (*10*), antitumoral (*11*), ethanol intake inhibition (*12*), and apoptosis-inducing activities (*13*). Its most important, carefully validated use, however, is in the symptomatic treatment of mild to moderate depression, and its efficacy has been validated through a number of clinical trials and meta-analytical studies (*14*). As a consequence of the popularity gained and to sustain the massive market demand, a great effort has been directed toward management of field cultivation in order to maximize both gross yield and quantity of phytochemicals reputedly responsible for the plant's therapeutic properties (*15*).

Because the purported health effects and market value of this plant are strictly linked to the amounts of hypericins, hyperforins, and flavonoids present, it is of interest to determine which factors affect the presence of such phytochemicals in *H. perforatum*. Secondary metabolism of St. John's Wort, however, is known to vary as a consequence of a number of biotic and abiotic factors such as genetic pool (*16*); geographical origin (*17*); harvest time (*18*); CO_2 (*19*); water; light and nitrogen abundance (*20*, *21*); and exposure to elicitors (*22*), toxic contaminants (*23*), pathogens, and herbivores (*24*).

The objective of this study was therefore to evaluate the consequences of a first reported phytoplasma disease on the content and quality of the main phytochemicals (i.e., hypericins, flavonoids, and essential oil) of cultivated *H. perforatum*, to define the effects the disease has on the market value of the cultivated crop, and to contribute to quality assurance for medicinal plant reproductive materials.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography (HPLC) grade solvents, PCR reagents, and solvent were purchased from Sigma-Aldrich (Milan, Italy) with the sole exception of Taq polymerase, which was obtained from Polymed (Florence, Italy). Compounds used as references for HPLC analysis were purchased from Extrasynthese (Genay, France), and references for gas chromatography (GC) and GC/MS were from Sigma-Aldrich, from both the "General" and the "Flavors and Fragrances" catalogues. However, the compounds labeled as tentatively identified in **Table 2** provided retention indices and mass spectra in good agreement with the literature (*25*). Water was purified by a Milli-Q_{plus} 185 system from Millipore (Milford, MA).

Plant Material. During the spring and summer of 2002, a phytoplasma infection was observed in an experimental field of *H. perforatum* L. (cvs. Godet Derbordance and Zorzi) located in Ozzano (Bologna, northern Italy) in its second year of cultivation. Starting in April, increasing percentages of plants were found showing severe symptoms: dwarfing, extreme reduction in leaf size, yellow leaves, proliferation of axillary buds (witches' broom), reduced internodal length, and lack of flower production. The majority of the symptoms (more than 50%) were observed in cv. Godet Derborance, which is normally dwarfed (~60 cm), but in this case, the plants were only 10−15 cm high. In the spring of 2003, during further investigations in the same experimental field, plants of Godet Derborance were not sprouting, while almost 20% of Zorzi showed symptoms similar to those previously noticed. In the summer of 2003, symptomatic and asymptomatic Zorzi plants were labeled after visual inspection of their aerial parts, individually tested for phytoplasma by nested PCR, collected, and dried in a dark greenhouse until extraction. About 1 kg of material was accurately weighed from the two batches (healthy and phytoplasma infected). The soil was fertilized by adding ripe cattle dung before the planting and in the autumn (each year, during cultivation). No insecticides or herbicides were used.

Phytoplasma Identification. To detect phytoplasmas, PCR tests were carried out with DNAs extracted from 1 g of *H. perforatum* tissues (leaflets and stem) using a chloroform/phenol procedure (*26*). DNAs were extracted from both symptomatic and asymptomatic plants and from control periwinkle samples. Nucleic acid (0.8 μL), suspended in a TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (pH 8.0)] and diluted to obtain a final concentration of 20 ng/μL, was added to a reaction mixture (25 μL total volume) containing 200 μmol of dNTP, 0.8 U of Taq polymerase (Polymed), 0.4 μmol of each primer, 10 mM TRIS buffer (pH 8.3), 50 mM HCl, 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin as the PCR buffer. The primer pairs employed were P1/P7 (*27*) followed in nested PCR by R16F2/R2 (*28*); 35 PCR cycles were conducted in an automated thermal cycler following the procedure described by Schaff et al. (*29*). Tubes containing the reaction mixture without DNA templates were included as negative controls. The PCR products (6 μL) were detected by 1% agarose gel electrophoresis followed by ethidium bromide staining and UV observation. In RFLP (restriction fragment length polymorphism) analyses, 100−200 ng of R16F2/R2 product (about 1240 bp) was digested with *Tru*I at 65 °C for at least 16 h following the manufacturer's instructions (Fermentas, Vilnius, Lithuania). The restriction patterns were compared with those of phytoplasma control strains after electrophoresis on a 5% polyacrylamide gel, ethidium bromide stained, and photographed under UV at 312 nm using a transilluminator.

Extractions. Dried *H. perforatum* flowering tops (about 0.5 g) were extracted twice with 100 mL of methanol at room temperature by sonication for 15 min. After filtration, the extracts were combined and concentrated at 40 °C under reduced pressure. The residue was dissolved in methanol and collected in a 25 mL volumetric flask, and solvent was added to make the final volume. All extracts and standard solutions were filtered through a 0.45 mm PTFE filter into a HPLC vial and capped. For each sample, the extraction procedure was carried out twice.

HPLC Conditions. The HPLC system consisted of an Agilent Technologies (Waldbronn, Germany) modular model 1100 HPLC system with an Agilent ChemStation for LC system (Rev. A.08.03). The detection was performed with a photodiode array detector working in the 200−650 nm range. Analyses were carried out on a Lichrospher

RP-18 column (125 mm × 4 mm i.d., 5 μm, Agilent Technologies) at room temperature. To protect the integrity of the analytical column, all analyses were performed with a coupled Lichrospher RP-18 guard column (4 mm × 4 mm, 5 μm, Agilent Technologies). As described by Brolis et al. (*30*), the mobile phase was (A) aqueous phosphoric acid (85%) solution (99.7: 0.3 v/v), (B) acetonitrile, and (C) methanol. The gradient elution was modified as follows: initial 100% A, 85% A and 15% B in 10 min; 70% A, 20% B, and 10% C in 30 min; 10% A, 75% B, and 15% C in 40 min; 5% A, 80% B, and 15% C in 55 min; and 100% A in 56 min. The total running time was 65 min. The flow rate was 1 mL/min. The detector monitored the eluent at 270 nm. The sample injection volume was 5 μL, and three injections were performed for each sample. Peaks were identified on the basis of their retention time (t_R) values and UV spectra by comparison with those of the standard component solution. The peak identity was also confirmed by spiking the extracts with pure standards.

Essential Oil Extraction. Steam distillation was used to extract *H. perforatum* essential oil from the dried flowering tops using a commercial Clevenger apparatus. After 6 h of steam distillation, 0.67 and 0.44 mL (yield 0.75 ± 0.21 and 0.11 ± 0.03%) of essential oil were obtained from 900 g of raw drug from healthy and infected plants, respectively. The essential oil content was determined on a volume to dry weight basis. The essential oil samples were dried over anhydrous sodium sulfate and stored in glass vials with Teflon-sealed caps at −18 ± 0.5 °C in the absence of light.

GC Analysis. Essential oil samples were analyzed, and the relative percentages were determined using a Fisons (Rodano, Milan, Italy) 9130-9000 series gas chromatograph equipped with a Fisons EL980 processor, a flame ionization detector (FID) detector, and a MEGA SE52 (Mega, Legnano, Italy) poly-5% diphenyl−95% dimethyl siloxane bonded phase column (i.d. = 0.32 mm, length = 30 m, and film thickness = 0.15 μm). Operating conditions were as follows: injector temperature, 280 °C; FID temperature, 280 °C; carrier (helium) flow rate, 2 mL/min; and split ratio, 1:40. The oven temperature was initially 45 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min, and finally held at that temperature for 10 min. One microliter of each sample dissolved (1:100 v/v) in CH_2Cl_2 was injected. The percentage composition of the oils was computed from the GC peak areas using the normalization method without any correction factors.

GC/Mass Spectrometry Analysis. Essential oil constituents were then analyzed by a Hewlett-Packard HP5890 series II plus gas chromatograph equipped with a HPMS 5989b mass spectrometer using electron impact and hooked up to the NBS75K library. The constituents of the volatile oils were identified by comparing their GC retention times, KI, and MS fragmentation patterns with those of other essential oils of known composition with pure compounds and by matching the MS fragmentation patterns and retention indices with the above-mentioned mass spectra libraries and with those in the literature (*25*). The GC conditions were the same as those reported for GC analysis, and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 40 μA; scan rate, 1 scan/s; mass range, 35−300 Da; and ion source temperature, 200 °C. A mixture of aliphatic hydrocarbons (C_8−C_{24}) in hexane (Sigma) was injected under the above temperature program to calculate the retention indices using the generalized equation by Van del Dool and Kratz (*31*).

RESULTS AND DISCUSSION

Phytoplasma Infection. The symptoms observed in 2003 on Zorzi plants were similar to those reported for *H. perforatum* Godet Derborance infected by phytoplasmas of the stolbur class (*32*). Specific direct and nested PCR as well as RFLP analyses revealed, for the first time in this species, the presence of phytoplasmas identified as belonging to ribosomal group 16SrVII (ash yellows). It must also be pointed out that some of the asymptomatic individuals of Zorzi gave positive results in PCR and RFLP assays.

Given that *H. perforatum* quality and efficacy are not strictly related to the amount of any single substance, the whole phytocomplex of cv. Zorzi was evaluated and data regarding essential oil profile and hypericins and flavonoid variations were collected.

Table 1. Quantitative Analysis of Methanolic Extracts of Healthy and Phytoplasma Infected *H. perforatum* Plants

compound	mg/g[a] healthy	mg/g[a] infected
chlorogenic acid	0.77 ± 0.02	1.56 ± 0.11
rutin	4.69 ± 0.02	1.96 ± 0.23
hyperoside	3.04 ± 0.05	2.38 ± 0.21
isoquercitrin	3.50 ± 0.08	1.47 ± 0.04
quercitrin	0.75 ± 0.03	0.72 ± 0.05
quercetin	0.18 ± 0.01	0.08 ± 0.01
amentoflavone	0.39 ± 0.02	0.12 ± 0.01
pseudohypericin	2.29 ± 0.07	1.41 ± 0.23
hypericin	1.36 ± 0.05	1.45 ± 0.01

[a] Data are expressed as means ± SD. For each sample, $n = 6$.

Methanolic Extracts. RP-HPLC analysis of the methanol extracts revealed a significant decrease in the abundance of flavonoids in *H. perforatum* infected plants. Healthy individuals, in fact, showed a total flavonoid content of 12.55 ± 0.31 mg/g, whereas in diseased individuals it was 6.74 ± 0.55 mg/g (**Table 1**). The abundance of rutin and isoquercitrin (4.69 ± 0.02 and 3.50 ± 0.08 mg/g, respectively) was remarkably different in phytoplasma-infected samples (1.96 ± 0.23 and 1.47 ± 0.04 mg/g, respectively). The overall naphthodianthrone content was mainly affected by the lower presence of pseudohypericin in diseased plants (1.41 ± 0.23 vs 2.29 ± 0.07 mg/g), while the variation in hypericin was imperceptible. A different trend was, instead, evidenced by chlorogenic acid, the presence of which was markedly increased in diseased *H. perforatum* (going from the 0.77 ± 0.02 mg/g in healthy plants to 1.56 ± 0.11 mg/g). As previously reported (*33*), host resistance may develop specific variations in the secondary metabolism of challenged tissues, inhibiting the flavonoid biosynthetic pathway and increasing the biosynthesis of caffeic and cinnamic derivatives. The net results provided would fit with these hypotheses.

Essential Oil Composition. After steam distillation, the aerial parts of healthy and phytoplasma-infected *H. perforatum* plants produced a pale oil at a yield of 0.75 and 0.11%, respectively. A quantitative decrease in excess of 60% was induced in phytoplasma-infected plants. Although the qualitative profile of both samples was very similar, their relative abundance was slightly different (**Table 2**). A total of 59 and 55 components were characterized, accounting for 94.7 and 92.9% of the essential oil in infected and healthy *H. perforatum*, respectively. The main components were sesquiterpenes: β-caryophyllene, germacrene D, β-copaene, γ-amorphene, and α-humulene, and overall results are in agreement with the results that report sesquiterpenes as the main constituents (*6*). The presence of the disease, however, induced a further enrichment (from 63.52 to 75.01%, a relative increase of 12.7%) of the entire sesquiterpenic class as compared to the abundance in healthy samples. Conversely, a matching decrease in monoterpene hydrocarbons (from 8.63 to 4.14%, a relative decrease of 51.20%) and aliphatics (from 6.31 to 2.58%, a relative decrease of 59.00%) was observed in the infected plants (**Table 2**). Spathulenol was detected in the one healthy sample (0.92%), and it was absent from the oil obtained from diseased plants. Such behavior seems to fit with the consequences of drought stress in essential oil-bearing plants (*34*) and could be related with phloem necrosis typically caused by phytoplasmas (*2*).

Table 2. Essential Oil Composition of Healthy and Phytoplasma-Infected *H. perforatum*

	compound[a]	KI	RA%[e] infected	RA%[e] healthy
1	octane, 2-methyl[c,d]	855	1.04	2.61
2	2,4-octadienal[c,d]	863	*f*	0.07
3	*n*-nonane[c,d]	901	0.25	0.56
4	α-thujene[c,d]	930	0.10	0.20
5	α-pinene[b]	938	0.42	0.81
6	β-pinene[b]	978	1.25	2.91
7	myrcene[b]	991	0.22	0.48
8	α-phellandrene[c,d]	1003	Tr	*f*
9	*cis*-3-hexenyl acetate[c,d]	1005	Tr	*f*
10	α-terpinene[b]	1017	0.07	0.11
11	*p*-cymene[c,d]	1024	Tr	0.13
12	β-phellandrene[b]	1030	0.18	0.39
13	*cis*-ocimene[c,d]	1037	0.07	0.15
14	*trans*-ocimene[c,d]	1051	1.42	2.67
15	γ-terpinene[b]	1060	0.15	0.27
16	decane, 2-methyl[c,d]	1071	0.07	0.23
17	terpinolene[b]	1089	0.05	0.10
18	*n*-undecane[c,d]	1100	0.16	0.23
19	nonanal[c,d]	1101	0.05	0.06
20	*allo*-ocimene[c,d]	1132	0.21	0.41
21	camphor[b]	1145	0.05	*f*
22	unidentified hydrocarbon	1176	Tr	0.37
23	terpinen-4-ol[b]	1177	0.34	Tr
24	α-terpineol[b]	1189	0.05	0.06
25	*n*-decanal[c,d]	1202	0.16	0.17
26	terpinen-4-ol acetate[c,d]	1300	0.09	0.14
27	elemene isomer[c]	1329	0.52	0.55
28	δ-elemene[b]	1339	4.58	3.86
39	α-longipinene[c,d]	1353	0.14	0.13
30	α-ylangene[c,d]	1373	0.05	0.11
31	α-copaene[c,d]	1377	0.10	0.16
32	β-bourbonene[b]	1388	0.31	0.50
33	β-elemene[c,d]	1391	0.31	0.64
34	β-funebrene[c,d]	1416	0.52	0.44
35	caryophyllene[b]	1421	30.09	25.41
36	β-copaene[b]	1433	7.09	5.97
37	aromadendrene[c,d]	1441	0.05	0.05
38	α-humulene[b]	1456	5.24	4.34
39	β-farnesene[b]	1458	2.26	1.39
40	germacrene D[b]	1487	19.64	17.24
41	γ-amorphene[c,d]	1496	6.28	5.23
42	α-muurolene[c,d]	1500	0.71	0.39
43	α-farnesene[c,d]	1506	1.66	1.69
44	δ-cadinene[c,d]	1523	1.60	1.55
45	cadina-1(2)4-diene trans[c,d]	1535	0.09	0.10
46	α-cadinene[c,d]	1538	0.12	0.16
47	*cis*-nerolidol[c,d]	1562	0.87	0.85
48	*cis*-3-hexenyl benzoate[c,d]	1567	0.14	0.17
49	germacrene-D-4-ol[c,d]	1576	0.27	*f*
50	spathulenol[b]	1579	*f*	0.92
51	caryophyllene oxyde[c,d]	1584	0.38	0.56
52	1,10-diepicubenol[c,d]	1618	0.22	*f*
53	epicubenol[c,d]	1629	0.54	0.58
54	cyclododecane + τ-cadinol[c,d]	1641	1.57	2.09
55	α-cadinol[c,d]	1654	0.61	0.87
56	cyclotetradecane + cadalene[c,d]	1677	1.10	1.73
57	α-bisabolol[b]	1686	0.26	0.21
58	*cis,cis*-farnesol[c,d]	1718	0.11	*f*
59	cyclohexadiene[c,d]		0.18	*f*
60	hexadecanol[c,d]	1876	*f*	0.27
61	phytol[c,d]	1943	0.37	1.41
62	eicosane[c,d]	1999	0.34	0.16
	monoterpene hydrocarbons		4.14	8.63
	oxygenated monoterpenes		0.53	0.20
	sesquiterpene hydrocarbons		75.01	63.52
	oxygenated sesquiterpenes		5.82	7.81
	aliphatic compounds		2.58	6.31
	total		94.72	92.86
	extraction yield		0.11	0.75

[a] Compounds are listed in order of elution from a SE-52 column. [b] Identified on the basis of comparison with MS database spectra, retention indices, and pure reference compounds. [c] Tentatively identified on the basis of comparison with MS database spectra and retention indices. [d] Identification based on a very good match of mass spectra. [e] RA%, relative area percentage. [f] Not detected.

Phytoplasma disease could thus become an issue in defining the market value for the final cultivation product of *H. perforatum* cultivated plants. Consequently, the phytopathological status of *H. perforatum* propagation material must be carefully controlled and such controls should be included in the guidelines for good agricultural practice. Considering that insecticides are not currently used to protect medicinal plants from these pathogens, new and adequate agronomical techniques must be adopted to prevent phytoplasma diseases from spreading.

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