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The effect of starch and incubation temperature in anther culture of potato

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Abstract

Three Andean tetraploid potato genotypes (2n = 48) and 7 anther-derived dihaploids (2n = 24) originating from two of the tetraploids were used in anther culture. Relative number of embryos/vial was significantly higher when the anther culture media was gelatinized with 3% potato starch than when Gelrite or wheat starch (3%) were used as gelatinizing agents. The degree of anther culture response varied between tetraploids but also within a group of related dihaploids. Additionally, the embryo production of individual genotypes, tetraploids as well as dihaploids, was dependent on the incubation temperature (10, 15, 20, 25, 30°C) of the anther culture. The incubation temperature of the anther culture was also important for the regeneration rate. Direct regeneration was mostly stimulated when the anther culture was incubated at 20°C.

Abbreviations: BAP – 6-benzylaminopurine, IAA – indole-3-acetic acid

Introduction

Anther culture is not frequently used in potato breeding due to genotypically dependent differences in haploid production. The problem can be overcome by genetic transfer of androgenetic capacity (Singsit & Veilleux 1989; Sonnino et al. 1989; Uhrig 1983; Uhrig & Salamini 1987), which is complicated and time consuming, however. Therefore, further investigations of methods to increase anther culture efficiency in less responsive genotypes is important.

Sorvari (1986a,b) demonstrated that addition of different types of starch to the medium, as the gelatinizing agent, had pronounced effects on anther culture of barley, as well as in regeneration of potato tuber discs. Barley and wheat starch showed the highest effect and were, according to Sorvari (1986a) and Sorvari & Schieder (1987), superior to agar as gelatinizing agents for anther culture of barley. By combining barley starch with maltose, Kuhlmann & Foroughi-Wehr (1989) optimized the procedure for barley anther culture to such an extent that it may be used economically. Calleberg et al. (1989) demonstrated increased embryo production when using potato starch as a gelatinizing agent in anther cultures of potato. Additionally, corn starch was used in tissue culture of tobacco and carrot and an increasing growth rate was seen (Henderson & Kinersley 1988).

The effect of culture temperature on embryogenesis in potato anther culture, and particularly on regeneration has not been extensively studied. Lillo (1989) and M'Ribu & Veilleux (1990) demonstrated, however, that temperature

was important for the regeneration of protoplast-derived callus and explants of potato.

The purpose of this investigation was to enhance embryogenesis and regeneration in anther culture of potato by using starch as a gelatinizing agent in the anther culture media. Secondly, we also wanted to investigate the effect of temperature, for both embryo production and regeneration.

Materials and methods

Plant material

Three tetraploid genotypes (199.13, 201.5, 201.12) (2n = 48) from the breeding programme for frost resistance at the International Potato Center (CIP) Lima, Peru (Landeo 1980), and 7 dihaploids (2n = 24) originating from anther cultures of the tetraploids 199.13 and 201.12 were used. The material from CIP has a background in several tuber bearing *Solanum* species, *S. phureja*, *S. stenotomum*, *S. curtilobum*, *S. ajanhuiri* and *S. tuberosum* ssp. *andigena*, as well as *S. tuberosum* material from South America. Details of the pedigree of the genotypes are given by Kristjansdottir (1989).

Anther culture procedure

Tubers were planted in a greenhouse (20°C ± 5°C) in January and the first flower buds emerged in March. Buds with pollen at the late tetrad or uninucleate stage were harvested and thereafter cold treated (4°C) for 3–4 days wrapped in aluminum foil. The flower buds were surface sterilized with 70% ethanol for 2 minutes and the anthers were inoculated aseptically on 5 cm Petri dishes (10 anthers per dish). The Petri dishes were sealed with Nescofilm.

Anther culture media

MS medium (Murashige & Skoog 1962) containing 6.0% sucrose and 45.0 mg l^{-1} L-cysteine-HCl (Merck, biopur) and the double layer technique (Johansson et a. 1982) were used in all experiments. The solid phase of the double layer media, however, was modified in that Gelrite (0.35%) (Merck & Co. Inc. (Rahway, NJ), Kelco Div, USA) in most experiments was combined with potato starch (3%) (Merck, FR Germany). In certain experiments, Gelrite was combined with potato starch (1%) or wheat starch (1 or 3%) (Kebo lab AB, Sweden). Starch, Gelrite, and 0.5% activated charcoal (Merck, FR Germany) were mixed in cold distilled water and heated until the mixture became viscous and subsequently autoclaved at 120°C for 1 h. Immediately after autoclaving, the hot liquid was mixed with filter-sterilized culture medium and poured (≈4 ml) into 5 cm Petri dishes. When solid, 4 ml of liquid medium was poured on top of it.

Incubation and examination

Incubation of the anther cultures was at a temperature of 10, 15, 20, 25 or 30°C in a 16-h day light regime (7 Wm^{-2}). Experiments, where the concentrations of starch and Gelrite varied, were incubated at 25°C exclusively. The number of calli and embryos were counted after 30, 60, and 90 days incubation at 10, 15, or 20°C. When incubation was at 25 and 30°C, the cultures were examined after 30 and 60 days.

Regeneration of plants

After 60–70 days in culture, the embryos were transfered to a double layer, K3 medium (Johansson 1988; Nagy & Maliga 1976) containing 1% sucrose 1 mg l^{-1} BAP, 1 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, and 1.5 mg l^{-1} Thidiazuron (Sheering, France). The solid phase (5 ml in a 5 cm Petri dish) was gelatinized with 0.4% Gelrite. Two ml liquid media were poured no top of the solid phase. The cultures were incubated in 25°C in a 16-h light regime (7 Wm^{-2}).

Statistical calculations

Statistical calculations were performed in, and visualized by, the JMP program for the Apple Macintosh computer. The homogenity of means were tested with analysis of variance. Comparison of individual means were performed with the method of LSD (Least Significant Difference) and grafically illustrated with comparison

circles. Means are significantly different when angle between circles is less than 90°.

Results

Relative number of embryos/vial in various gelatinizing agents

Relative number of embryos/vial in anther cultures, where Gelrite, potato starch (1 and 3%) or wheat starch (1 and 3%) were used to gelatinize the media, were compared (Fig. 1). After 30 days of culture, relative number of embryos/vial in potato starch (3%) and in wheat starch (1%) were similar and significantly higher than wheat starch (3%) and Gelrite. After 60 days, the relative number of embryos/vial in potato starch (3%) was highest and significantly higher than wheat starch (3%) and Gelrite. The optimum starch concentrations, among the concentrations tested, were 3% for potato starch and 1% for wheat starch.

Relative number of embryos/vial at various incubation temperatures

Relative number of embryos/vial in anther culture of the tetraploid 199.13 increased with increasing incubation temperature. The tetraploid 201.5 produced similar amounts of embryos in 20 and 30°C while the tetraploid 201.12 produced the highest relative number of embryos/vial in 25°C (Fig. 2). The embryo production patterns, due to various incubation temperatures, in dihaploids and their tetraploid mothers differed in some cases. Of the six dihaploids derived from the tetraploid 199.13, three (HA 13.1, HA 13.7, HA 13.11) showed a pattern similar to 199.13, while HA 13.2 and HA 13.4 produced more embryos in 20 and 25°C. HA 13.9 demonstrated a scattered embryo production pattern including peaks at 15 and 30°C. The embryo production optimum of HA 12.2 was 15°C rather than 25°C, as was the case for the tetraploid mother 201.12.

Regeneration at various incubation temperatures

Embryos of genotypes 199.13, 201.5, 201.12, HA 13.1, HA 13.9, and HA 12.2 regenerated directly on the anther culture media. The highest production of shoots/embryo occured when anther cultures were incubated at 20°C (Table 1). No direct regeneration occured at 30°C despite a high embryo production in some genotypes. The production of shoots/embryo at 20°C was significantly higher than at 10 and 30°C. Shoots/embryos transfered to a regeneration medium,

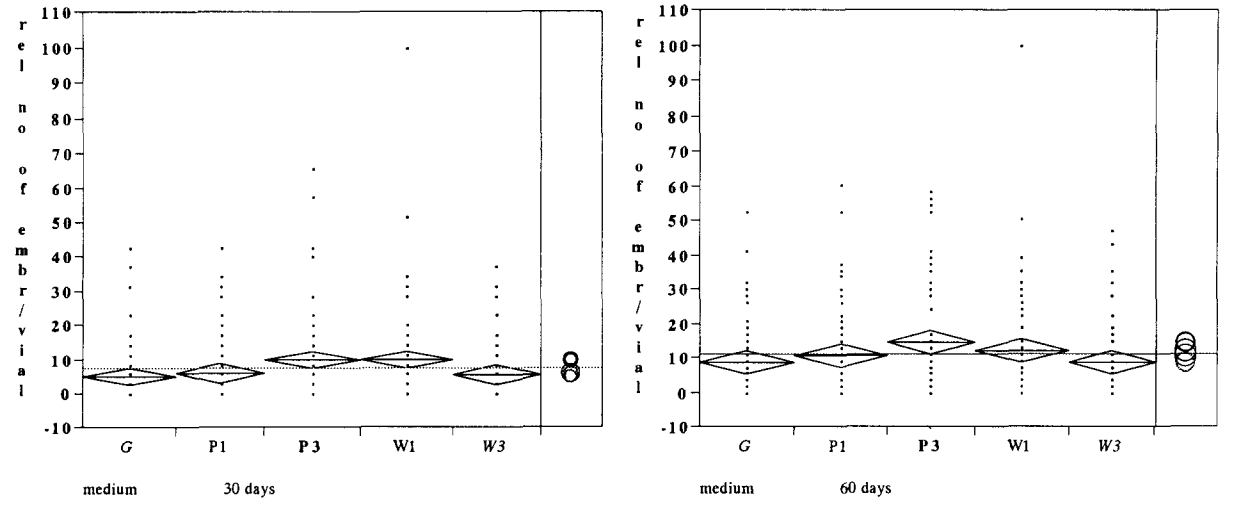

Fig. 1. Relative number of embryos/vial in anther cultures where different gelatinizing agents were tested (G = Gelrite, P1 = 1% potato starch, P3 = 3% potato starch, W1 = 1% wheat starch, W3 = 3% wheat starch). 58-66 vials (represented by dots) of genotype 199.13 were analyzed in each medium. The homogenity of means were tested with analysis of variance (30 days: DF = 4, 312; F = 2,61; $p < 0.04$, 60 days: DF = 4, 299; F = 1,93; $P < 0,10$). Means with 95% confidence interval (represented by lozenges). Means comparison circles, 95%.

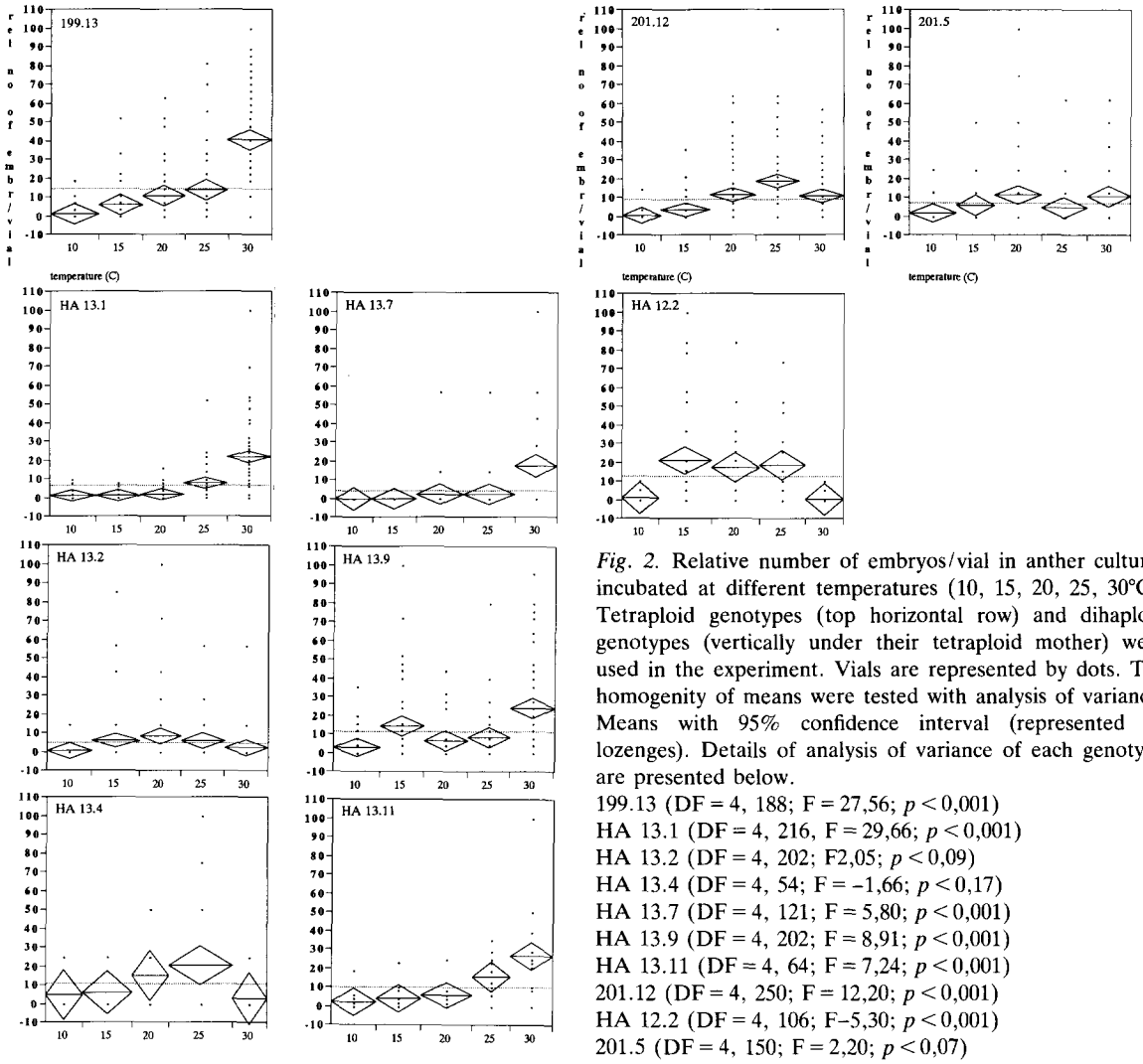

Fig. 2. Relative number of embryos/vial in anther cultures incubated at different temperatures (10, 15, 20, 25, 30°C). Tetraploid genotypes (top horizontal row) and dihaploid genotypes (vertically under their tetraploid mother) were used in the experiment. Vials are represented by dots. The homogenity of means were tested with analysis of variance. Means with 95% confidence interval (represented by lozenges). Details of analysis of variance of each genotype are presented below.
199.13 (DF = 4, 188; F = 27,56; $p < 0{,}001$)
HA 13.1 (DF = 4, 216, F = 29,66; $p < 0{,}001$)
HA 13.2 (DF = 4, 202; F2,05; $p < 0{,}09$)
HA 13.4 (DF = 4, 54; F = –1,66; $p < 0{,}17$)
HA 13.7 (DF = 4, 121; F = 5,80; $p < 0{,}001$)
HA 13.9 (DF = 4, 202; F = 8,91; $p < 0{,}001$)
HA 13.11 (DF = 4, 64; F = 7,24; $p < 0{,}001$)
201.12 (DF = 4, 250; F = 12,20; $p < 0{,}001$)
HA 12.2 (DF = 4, 106; F–5,30; $p < 0{,}001$)
201.5 (DF = 4, 150; F = 2,20; $p < 0{,}07$)

was independent of the incubation temperature of the anther culture. No significant difference was found between means of all genotypes in different temperatures (Table 2). Genotypic differences in regeneration capacity were also found.

Discussion

In experiments performed by Calleberg et al. (1989), 3% potato starch was used as the gelatinizing agent. Embryo production was then significantly higher in potato starch compared to Gelrite at the beginning of the culture period. However, after additional culture time, the embryo frequency in the medium with Gelrite was significantly higher. A plausible explanation of the latter may be an enzymatic breakdown of starch (Sorvari 1986a), which, according to Calleberg et al. (1989), resulted in a release of activated charcoal and residues of starch into the liquid phase of the culture media. The starch breakdown products as well as the presence of unbound activated charcoal probably diminished the availability of oxygen as well as light in the culture, and, as a result, embryo production was hampered. Since embryo production obviously was stimulated in the presence of potato starch, prior to the changes of the media, we tried to improve the method. Sorvari (1986a) avoided the structural problem by placing a nylon net on

Table 1. Direct regeneration (shoots/embryo) of embryos produced in anther cultures incubated at 5 different temperatures.

Temp.(C)	10				15				20			
Genotype	anthers	embryos	shoots	shoots/embr.	anthers	embryos	shoots	shoots/embr.	anthers	embryos	shoots	shoots/embr.
199.13	420	17	0	0	380	63	0	0	370	107	5	0,047
HA 13.1	460	22	0	0	440	25	0	0	440	38	1	0,026
HA 13.9	440	34	3	0.088	410	149	6	0.04	410	70	6	0,086
201.12	440	8	0	0	570	61	2	0,033	530	174	15	0,086
HA 12.2	190	5	0	0	260	105	1	0,009	240	80	2	0,025
201.5	330	5	0	0	270	13	1	0,077	330	31	1	0,032
			mean =	0,015 a			mean =	0,026 ab			mean =	0,05 b
			S.D. =	0,036			S.D. =	0,03			S.D. =	0,029

Temp.(C)	25				30			
Genotype	anthers	embryos	shoots	shoots/embr.	anthers	embryos	shoots	shoots/embr.
199.13	380	139	4	0,029	380	419	0	0
HA 13.1	430	170	2	0,012	430	480	0	0
HA 13.9	400	86	3	0,035	410	251	0	0
201.12	500	277	5	0,018	510	158	0	0
HA 12.2	230	81	3	0,037	190	3	0	0
201.5	320	13	0	0	300	26	0	0
			mean =	0,022 ab			mean =	0,0 a
			S.D. =	0,014			S.D. =	0

Letters indicate means which differ significantly (least significant difference, $p < 0{,}05$).
Six genotypes were used in the experiment. The homogenity of the means were tested with analysis of variance (DF = 4, 25; F = 3,16; $p < 0{,}03$).

Table 2. Regeneration of embryos (shoots/embryo) transfered to a regeneration medium.

Temp.(C)	10			15			20		
Genotype	embryos	shoots	shoots/embr.	embryos	shoots	shoots/embr.	embryos	shoots	shoots/embr.
199.13	17	0	0	23	0	0	29	2	0,069
HA 13.1	19	2	0,105	25	0	0	16	0	0
HA 13.9	18	2	0,111	27	4	0,148	20	2	0,1
201.12	8	0	0	32	0	0	30	0	0
HA 12.2	5	0	0	39	0	0	33	0	0
201.5	3	0	0	4	0	0	4	0	0
		mean =	0,036 a		mean =	0,025 a		mean =	0,028 a
		S.D. =	0,056		S.D. =	0,06		S.D. =	0,045
Temp.(C)	25			30					
Genotype	embryos	shoots	shoots/embr.	embryos	shoots	shoots/embr.			
199.13	45	1	0,022	20	0	0			
HA 13.1	49	0	0	42	2	0,048			
HA 13.9	30	0	0	80	0	0			
201.12	86	2	0,023	28	0	0			
HA 12.2	55	1	0,018	8	0	0			
201.5	8	0	0	9	1	0,111			
		mean =	0,01 a		mean =	0,026 a			
		S.D. =	0,012		S.D. =	0,046			

Embryos were produced in anther cultures incubated at 5 different temperatures.
Letters indicate means which differ significantly (least significant difference, $p < 0,05$).
Six genotypes were used in the experiment. The homogenity of the means were tested with analysis of variance (DF = 4, 25; F = 0,23; $p < 0,92$).

top of the medium. We found, however, that a mixture of starch and Gelrite was sufficient to keep the medium solid. As demonstrated in this paper, this modification resulted in a higher embryo production in the medium with potato starch compared to the medium with Gelrite only, throughout the entire culture period.

Starch has been compared to agar (Henderson & Kinnersley 1988; Sorvari 1986a,b; Sorvari & Schieder 1987) and its stimulating effect in tissue culture is often explained by the absence of inhibitory substances, which are present in agar (Johansson 1983; Kohlenbach & Wernicke 1978). In our investigation, we compared starch with Gelrite, an inert gel, which Johansson (1988) had shown to be superior to agar for shoot multiplication of potato. We therefore suggest that the functions of starch in our experiment may be of a nutritional and/or an osmotic nature in addition to the function as a gelling agent. The effect of potato starch (3%) in this investigation was more pronounced in embryo production than wheat starch, which indicates a species specific nutritive role. It is, however, difficult to claim such a relationship from these results, since the embryo production rate was shown to be dependent also on starch concentration.

Starch did not improve the regeneration rate in this investigation, in contrast to other studies, where starch improved the regeneration rate in barley anther culture (Kuhlmann & Foroughi-Wehr 1989; Sorvari 1986a; Sorvari & Schieder 1987), of potato tuber discs (Sorvari 1986b), and of tobacco and carrot explants (Henderson & Kinnersley 1988).

Anther culture capacity was found to be genotypically dependent, in agreement with many other investigations. The degree of anther culture response varied between tetraploids but also within a group of related dihaploids, which indicates a heterozygous condition of the androgenetic capacity in the tetraploids parent. The latter corresponds well with Veilleux et al. (1985) and Wenzel & Uhrig (1981), who noted dihaploids with both higher and lower androgenetic capacity compared to the tetraploid mother. Additionally, we observed that the em-

bryo production of individual genotypes, tetraploids as well as dihaploids was dependent on the incubation temperature. Consequently the variability for androgenetic capacity is also temperature dependent. Calleberg et al. (1989) demonstrated a temperature dependent relationship between androgenesis and pollen germination and suggested, based on results published by Kristjansdottir (1990), a correlation between temperature tolerance of the plant and its anther culture capacity in various temperatures. The amount of pollen germinated at different temperatures indicates, according to Kristjansdottir (1990), the temperature tolerance of a genotype.

Anther culture conditions seem to be important for regeneration as well. Batty & Dunwell (1989) showed that the composition of the anther culture medium substantially increased the regeneration rate. In several respects, this investigation points out the importance of incubation temperature. Some genotypes responded by producing a high number of embryos at 30°C, while others produced fewer. In both cases, no regeneration, directly on the anther culture medium, was seen. Several plausible explanations exist. Firstly, a high number of embryos may result in a disadvantageous surrounding, due to increased competition and development of harmful substances in the media (Johansson et al. 1990). Secondly, higher temperatures may speed up the ageing of the embryos, which shortens the germination period, and starts production of harmful substances at an early stage. Thirdly, high temperature may stimulate production of secondary embryos which, according to Johansson (unpublished), may lack a normal regeneration capacity. Lastly, high temperatures may induce a dormancy in embryos, which is not broken until embryos are transferred to regeneration media. According to our experiments, the highest regeneration rate, directly on the anther culture medium, was found when incubation of the anther culture took place at 20°C. Similar results were seen by Lillo (1989) and M'Ribu & Veilleux (1990), who found 20°C to be adventageous for regeneration of protoplast-derived callus of potato as well as potato explants.

Additionally, genotypic differences were found to influence the regeneration process separately from embryogenesis, which was also demonstrated by Cappadocia et al. (1984) and Johansson (1986). In correspondence with Fish & Jones (1988) and Coleman et al. (1990) who studied regeneration from explants, the regeneration capacity varied between dihaploids derived from the same tetraploid parents.

By transfering the embryos to a regeneration medium, it is possible to obtain a low frequency of shoots. The regeneration frequency was, in this experiment, independent of the incubation temperature of the anther culture. The presence of auxins in the regeneration media stimulates callus formation, possibly independent of status and origin of the embryo. Consequently, a callus phase is always present prior to regeneration in this case, as compared with direct regeneration on the anther culture media where germination most commonly occurs. To increase the regeneration rate many experiments have been done, mainly by manipulation of the regeneration media, but only with limited success. We suggest that more work should be done to find suitable anther culture conditions which enable direct regeneration. Firstly, in direct regeneration we avoid a callus phase which may be negative for chromosomal stability. Secondly, the total number of plants obtained in relation to the amount of work is of major importance, which points towards the less time-consuming inoculation of more anthers in a suitable condition, instead of the time-consuming transfering of embryos to a regeneration medium.

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