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CALLUS INDUCTION AND PLANT REGENERATION FROM MATURE EMBRYOS OF DIFFERENT WHEAT GENOTYPES

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Abstract

Mature embryos of 5 *Triticum aestivum* and 5 *T. durum* cultivars formed embryogenic callus on two different media. Embryos were removed from surface sterilised seeds and placed with the scutellum upwards on a solid agar medium containing the inorganic components of Murashige & Skoog and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) or 1 mg/L naphtalenacetic acid (NAA). The developed calli and regenerated plants were maintained on 2,4-D or NAA free MS medium. Wheat plants can be regenerated *via* two different systems. There were significant differences in percentage of callus induction and regeneration capacity on the different initiation medium. Among the *T.aestivum* cultivars, Yakar had the highest regeneration capacity in both induction medium. In *T.durum* cultivars, Kızıltan gave the highest regeneration capacity in MS+2,4 D medium and Yılmaz gave the highest regeneration capacity in MS+NAA medium. A strong genotypic effect on the culture responses was found for both induction medium.

Introductıon

The regeneration of whole plants from selected transgenic tissues is required for the successful application of biotechnology in crop improvement. Wheat is one of the most important species of food crop. Therefore, it has been extensively investigated with respect to plant regeneration from *In vitro* culture. Shoot regeneration is of crucial importance in the realization of the potential of cell and tissue culture techniques for plant improvement (Purnhauser *et al*., 1987).

Wheat has different regeneration systems such as somatic embryogenesis and *de novo* adventitious bud formation in *In vitro* tissue culture (Papenfus & Carman, 1987). Somatic embryogenesis has been observed in several gramineous species such as maize, barley, rice, bread and durum wheat (Benkirane *et al*., 2000). In wheat species, different explant sources have been used for embryogenic callus formation and plant regeneration: mature and immature embryos (Özgen *et al*., 1998; Özgen *et al*., 1996), infloroscences (Redway *et al*., 1990; Benkirane *et al*., 2000), coleoptile (Benkirane *et al*., 2000), shoot apical meristems (Ahmad *et al*., 2002) and anthers (Asrmstrong *et al*., 1987). These tissues vary in their ability to regenerate whole plants (Delporte *et al*., 2001). Immature embryos and immature infloroscences gave the highest frequencies of regenerated plants *In vitro* (Benkirane *et al*., 2000).

Tissue culture responses which includes callus induction and regeneration capacity of wheat are influenced by the genotypes, explant source, geographical origin, physiological status of the donor plants, the culture medium and the interactions between them (Özgen *et al*., 1996). In the present study, we report an efficient *In vitro* plant regeneration system from mature embryos of 10 wheat genotypes. We assesed the effect

of auxin type on the calli induction response and two regeneration system were compared. We demonstrated that calli derived from mature embryos have the good ability to undergo somatic embryogenesis and organogenesis.

Materials and Methods

The different genoytpes studied were as follows: for *T.aestivum*, 5 winter wheat cultivars (Gün 91, Yakar, İkizce, Mızrak, Uzunyayla); for *T. durum*, 5 winter wheat cultivars (Kızıltan, Altın, Yılmaz, Ç-1252, Ankara 98) seeds were obtained from Central Research Institute for Field Crops, Ankara Yenimahalle Campus.

Mature seeds were surface-sterilized with 20 % commercial bleach for 20 min., and then rinsed with sterilized distilled water, then they were left in 70 % ethanol for 3 min., followed by two changes of sterile distilled water. Seeds were randomized and mechanically vibrated during sterilization and rinsing. Seeds were imbibed in sterile distilled water for over night at room temperature. For callus induction, mature embryos were aseptically removed with a scalpel from the imbibed seeds. Ten embryos were cultured per sterile 90 mm Petri dishes.

For callus induction, the effect of two induction media were compared. Mature embryos were placed with the scutellum upwards on a solid agar medium in sterile Petri dishes and cultured for 14 days at 25 ± 1 ^{o}C under a 16 h photoperiod. The basal culture media consisted of the mineral salts of Murashige & Skoog (MS) supplemented with either 2 mg/L 2,4- dichlorophenoxyacetic acid (2,4-D) or 1 mg/L naphtalenacetic acid (NAA). Both media contained 30 g/L sucrose and were adjusted to pH 5.7, solidified with 7 g/L agar and autoclaved for 20 min., at 121^{o}C and 1.1 kg/cm^{2} pressure.

For shoot and root initiation, calli were transferred to MS/2 medium (MS with half-strength macronutrients) without growth regulators and cultured at 25 ± 1 ^{o}C in a 16h/8h light/dark cycle for 3-4 weeks. When roots and shoots were established, young plants were grown in test tubes containing the same medium. After two or three weeks, they were transplanted to soil in pots.

A completely randomised design with three replications per genotype was used. The effect of genotype and medium on culture responses were determined by analysis of variance and least significant-difference tests.

Results

Various genotypes of *Triticum aestivum* and *T. durum* were evaluated on two types of induction media. The first step for both induction media was callus induction from mature embryos. After that wheat plants could be regenerated from callus *via* two different regeneration systems. The first regeneration system was observed on a medium containing 2,4-D. In this system, wheat plants were regenerated *via* somatic embryogenesis from the callus. In the second regeneration system, plants were produced *via* adventitious shoots regeneration from the callus on a medium containing NAA.

Callus induction and plant regeneration from mature embryos of *T. aestivum* cultivars: Mature embryos formed embryogenic callus in both induction medium. Callus was first visible within two and three days in MS+2,4-D (2 mg/L) medium, MS+NAA (1mg/L) medium respectively. Callus induction rate, regeneration capacity and fresh weight of callus were greatly influenced by the genotype for both induction medium (Table 1).

Table 1. Embryo culture responses of five *Triticum aestivum* cultivars.

Medium	Genotype	Callus induction (%)	Regeneration capacity of callus (%)	Weight of callus (g)
MS+2,4D	Gün 91	41.67^{abc}	44.00^{cd}	0.0201^{c}
	Yakar	53.00^{ab}	63.67^{bc}	0.0206^{c}
	İkizce	57.67^{a}	21.67^{de}	0.0155^{c}
	Mızrak	45.33^{ab}	12.00^{e}	0.0178^{c}
	Uzunyayla	25.67^{c}	51.67^{bcd}	0.0222^{c}
MS+NAA	Gün 91	43.67^{ab}	55.00^{bc}	0.0512^{a}
	Yakar	48.33^{ab}	100.00^{a}	0.0577^{a}
	İkizce	48.00^{ab}	76.00^{ab}	0.0167^{c}
	Mızrak	46.66^{ab}	37.67^{cde}	0.0272^{bc}
	Uzunyayla	36.00^{bc}	58.00^{bc}	0.0444^{ab}

Data scored after 2 weeks (for callus induction and weight of callus) and 6 weeks (for regeneration capacity) in culture; 100 explants per treatment.
Means followed by the same letter are not significantly different at the 0.05 probability level

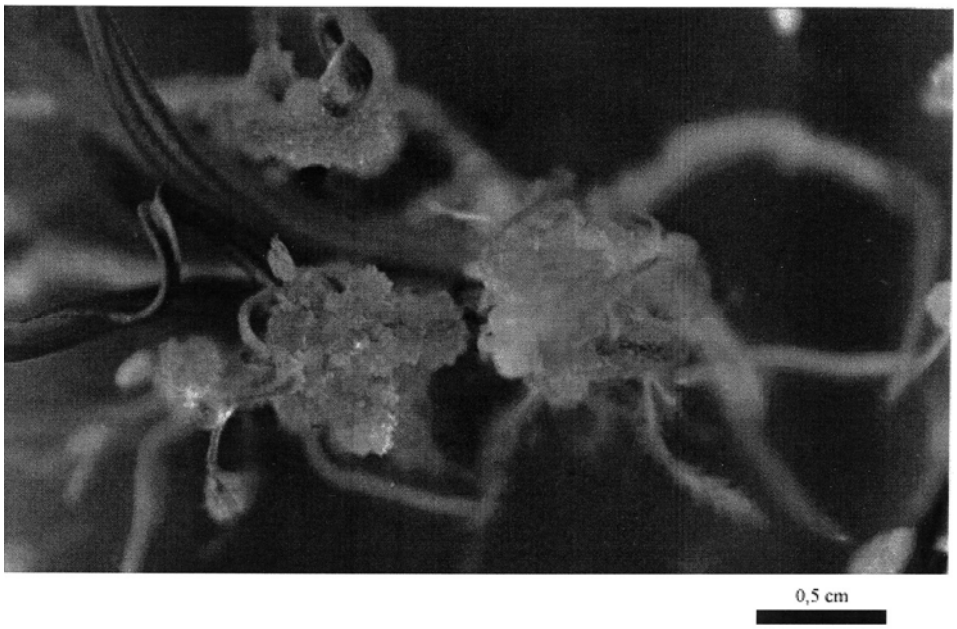

Fig. 1. Callus formation from *T. aestivum* cultivars in MS+NAA medium.

Callus induction frequencies varied from 36.00 % to 48.33 % depending on the cultivar in MS+NAA (1mg/L) medium (Fig. 1). Among the *T.aestivum* cultivars, Yakar had the highest callus induction frequency (48.33 %) and all of the calli were regenerable (100 %). Next to Yakar, İkizce was both for induction and regeneration ability. Uzunyayla produced the lowest callus induction frequency (36.00 %) but regeneration capacity of callus was relatively higher (58.0 %) than Mızrak (37.6 %) and Gün 91 (55.0 %).

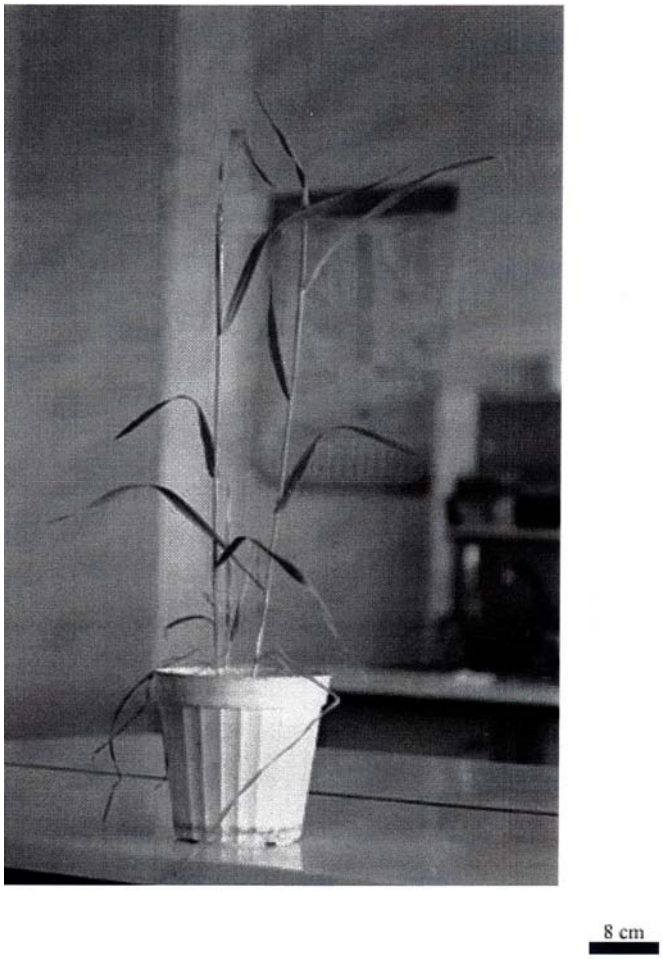

Fig. 2. Regenerated plant from *T. aestivum*.

In MS+2,4-D medium, genotype affected the callus formation and regeneration, apparently. Frequencies of callus induction varied from 25.6 % to 57.6 %. İkizce produced the highest callus induction frequency (57.6 %), but had a low regeneration capacity of callus (21.6 %). In the same medium, Yakar had relatively better induction and the highest regeneration capacity (63.67 %).

Mature embryo-derived embryogenic callus readily formed green shoots on the regeneration medium for both induction medium. Regeneration capacity was also affected by cultivar with variations from 12.00 % (Mızrak) to 63.67 % (Yakar) and from 37.6 % (Mızrak) to 100.00 % (Yakar) in MS+2,4-D and MS+NAA medium respectively (Fig. 2). The fresh weight of callus was higher in MS+NAA (1mg/L) medium than MS+2,4-D (2 mg/L) medium.

Statistical differences in percentages of callus induction (LSD=16.51 $p<0.05$), regeneration capacity (LSD=27.94 $p<0.05$) and fresh callus weight (LSD=0.0173 $p<0.05$ were found significant on the different initiation media.

Table 2. Embryo culture responses of five *Triticum durum* cultivars.

Medium	Genotype	Callus induction (%)	Regeneration capacity of callus (%)	Weight of callus (g)
MS+2,4D	Kızıltan	37.33^{c}	62.00^{bc}	0.0267^{ab}
	Altın	48.67^{ab}	27.67^{d}	0.0430^{a}
	Yılmaz	41.00^{abc}	52.33^{c}	0.0266^{ab}
	Ç-1252	43.33^{abc}	55.33^{c}	0.0254^{ab}
	Ankara 98	37.33^{c}	24.67^{d}	0.0311^{ab}
MS+NAA	Kızıltan	44.67^{abc}	63.33^{bc}	0.0120^{b}
	Altın	49.33^{a}	74.33^{ab}	0.0289^{b}
	Yılmaz	44.67^{abc}	82.33^{a}	0.0254^{ab}
	Ç-1252	42.33^{abc}	73.67^{ab}	0.0305^{ab}
	Ankara 98	39.67^{c}	81.67^{a}	0.0274^{ab}

Data scored after 2 weeks (for callus induction and weight of callus) and 6 weeks (for regeneration capacity) in culture; 100 explants per treatment.
Means followed by the same letter are not significantly different at the 0.05 probability level

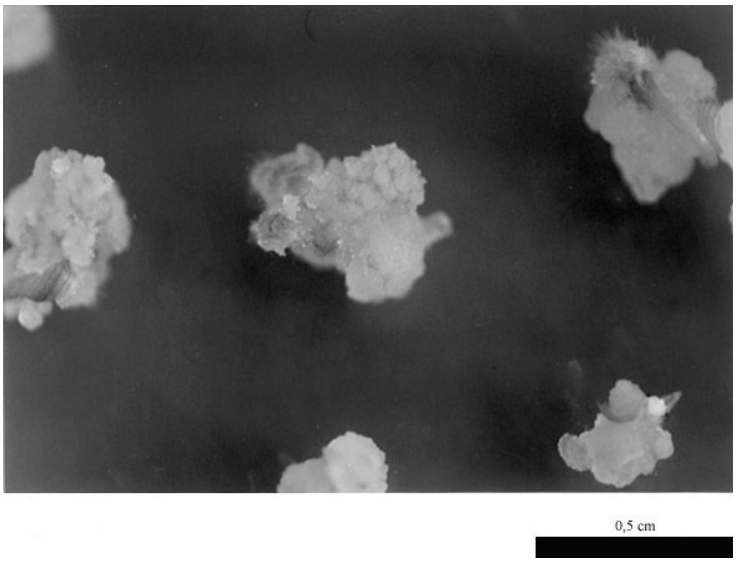

Fig. 3. Callus formation from *T. durum* cultivars in MS+2,4-D medium.

Callus induction and plant regeneration from mature embryos of *T. durum* cultivars: Callus formation was initiated after 4 days of culture in MS+NAA (1mg/L)medium and after 3 days of culture in MS+2,4-D (2mg/L) medium. The percentage of callus formation ranged from 37.33 % to 48.67 % and from 39.67 % to 49.33 % depending on the cultivars in MS+2,4-D and MS+NAA medium, respectively. Significant differences were observed in the callusing ability of the 5 cultivars tested for embryogenic callus formation using mature embryos (Table 2). In MS+2,4-D medium, Altın formed the highest percentage of callusing (48.67 %). Kızıltan and Ankara 98 had a low callus induction frequency (37.33 %) (Fig. 3). Similarly, Altın had the highest (49.33 %) and Ankara 98 had the lowest (39.67 %) callus induction frequency in MS+NAA medium.

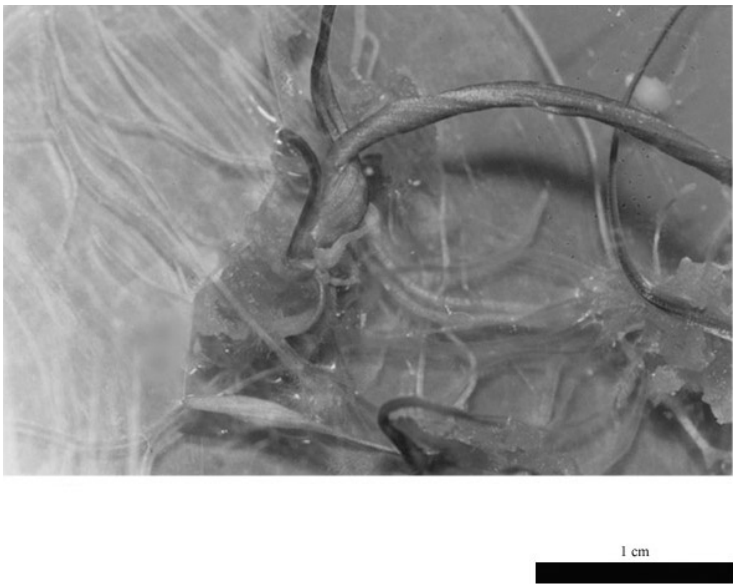

Fig. 4. Plant regeneration from *T. durum* cultivars in MS+NAA medium.

In MS+NAA medium, callus produced green leaves and shoots. Following transfer to the NAA free ½ MS medium, leaves elongated and plant regeneration were obtained (Fig. 4). In this medium, regeneration capacity varied from 63.33 % to 82.33 %. Yılmaz had an excellent (82.33 %) and Kızıltan had the lowest (63.33 %) regeneration capacity in this medium.

Calli with green spots produced somatic embryos (Fig. 5) in MS+2,4-D medium. After they were transfered to shoot initiation medium (2,4-D free ½ MS medium), somatic embryos developed shoots and leaves (Fig. 6).

Plant regeneration and production of multiple shoots from mature embryo calli were obtained after about 2 months from callus initiation. While Kızıltan had the highest regeneration capacity (62.0 %) in MS+2,4-D medium, Yılmaz had an excellent regeneration capacity (82.3 %) in MS+NAA medium. In both induction medium, callus induction, fresh weight of callus and regeneration capacity were greatly influenced by the genotype. There were significant differences in percentage of callus induction (LSD=8.08 $p<0.05$), fresh callus weight (LSD=8.08 $p<0.05$) and regeneration capacity (LSD=13,42 $p<0.05$) on the different initiation medium. For both *T.aestivum* and *T. durum* cultivars, the regeneration capacities in MS+NAA medium (70.20%) are higher than the regeneration capacities in MS+2,4-D medium (41.20 %).

Discussion

Shoot regeneration is a crucial importance in the realization of potential of cell and tissue culture techniques for plant improvement. Auxins which are essential for callus induction, play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration (Purnhauser *et al.,* 1987). The auxin 2,4-D is required for the production of somatic embryogenesis (Armstrong *et al.,* 1987) in cereals. Somatic embryos are formed on nutrient medium with a reduced 2,4-D concentration (Delporte *et al.,* 2001).

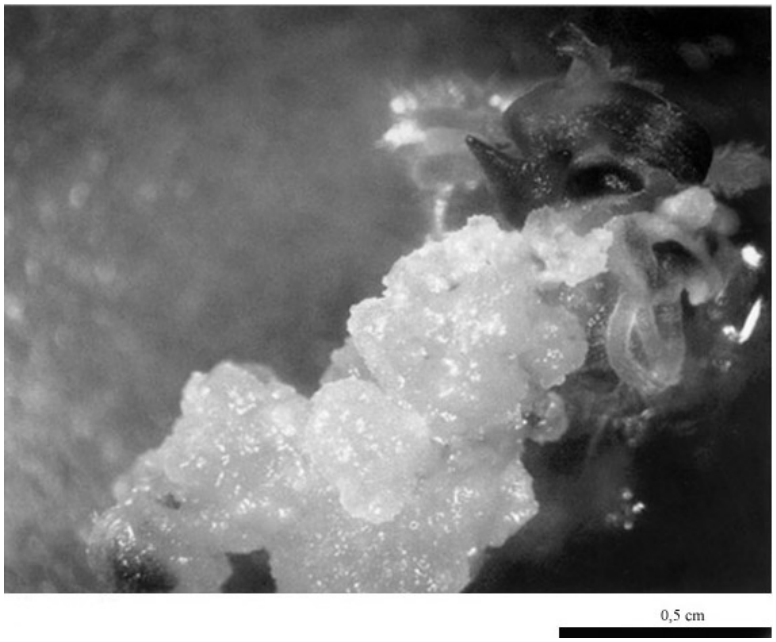

Fig. 5. Somatic embryogenesis on isolated embryo explants of wheat.

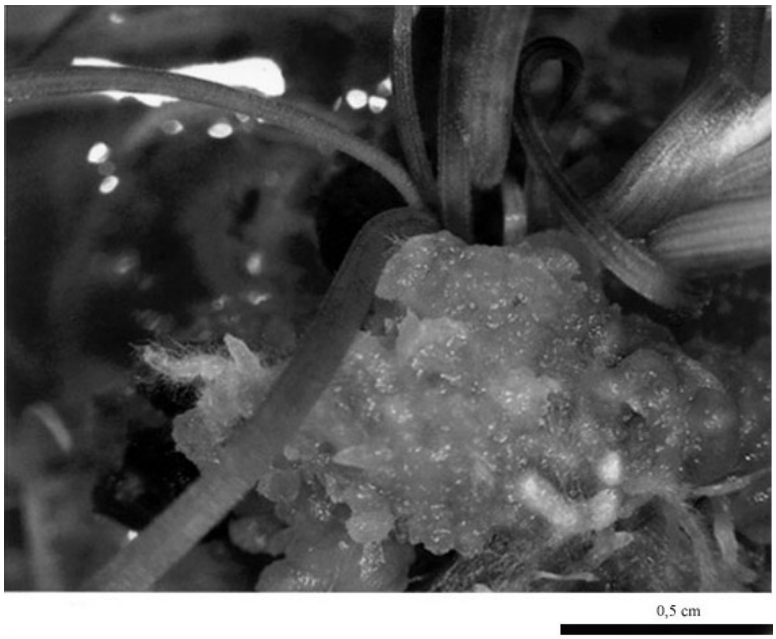

Fig. 6. Plant regeneration on the shoot initiation medium from *T. durum* cultivars.

In this study, two different auxins were assessed on the basis of the embryogenic calli induction. However our results revealed two different regeneration systems depending on auxin types. In MS+NAA (1mg/L) medium, the green centers were initiation of adventitious shoots and following transfer to shoot initiation medium, embryogenic callus rapidly developed shoots and leaves. This regeneration system produced a greater number of plants than the other regeneration system. Also, *In vitro* culture duration of the two regeneration systems varied significantly. While the first regeneration system (in MS+NAA) took 4-5 weeks to obtain plants ready for transfering to soil, the second regeneration system (in MS+2, 4-D) took 8-9 weeks. As it is clear, the first regeneration system is faster than second one.

Immature embryos are regarded as the most efficient tissue source for the highest frequencies of regenerable plants *In vitro* (Redway *et al*., 1990). However, they are available only for limited periods of the year. In contrast, mature embryos could be available at any time (Özgen *et al*., 1996; Özgen *et al*., 1998). The highest regeneration frequency obtained in the present study is 100% for *T. aestivum* cultivars. This result is consistent with the reports of Özgen *et al*., (1998). However Delporte *et al*., (2001) reported an average of 11% of regeneration capacity which is much lower than the present study for mature culture in their study. For *T. durum* cultivars, the highest regeneration frequency obtained in this study is 82.33% in MS+NAA medium and 62.00% in MS+2, 4-D medium. Similarly, Özgen *et al*., (1996) reported an average of 70.4% of regeneration capacity for mature embryo culture. Delporte *et al*., (2001) reported that 75% of the genotypes tested presented a regeneration frequency of less than 30% but only a few genotypes revealed a regeneration rate of upto 60%. There, our results are highly greater than this report.

In this study, genotype and medium had significant effects on regeneration capacity of *T.aestivum* and *T. durum* cultivars. Mature embryos being available throughout the year were found to be suitable explant in wheat tissue culture studies.

Acknowledgements

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