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Effects of mutagens on somatic embryogenesis and plant regeneration in groundnut

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Abstract

The embryogenic calli (EC) were obtained from hypocotyl explants of groundnut (*Arachis hypogaea* L.) cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg dm^{-3} 6-benzylaminopurine (BAP). The EC were exposed to γ-radiation (10 - 50 Gy) or treated with 1 - 5 mM of ethyl methane sulphonate (EMS) or sodium azide (SA). The mutated EC were subcultured on embryo induction medium containing 20 mg dm^{-3} 2,4-D. Somatic embryos (SE) developed from these calli were transferred to MS medium supplemented with BAP (2.0 mg dm^{-3}) and 0.5 mg dm^{-3} 2,4-D for maturation. The well-developed embryos were cultured on germination medium consisting of MS salts with 2.0 mg dm^{-3} BAP and 0.25 mg dm^{-3} naphthaleneacetic acid (NAA). Well-developed plantlets were transferred for hardening and hardened plants produced normal flowers and set viable seeds. The fresh mass of the EC, mean number of SE per explant and regeneration percentage were higher at lower concentrations of mutagens (up to 30 Gy/3 mM). Some abnormalities in regenerated plants were observed, especially variations in leaf shape.

Additional key words: *Arachis hypogaea*, chemical mutagens, embryogenic callus, γ-radiation, growth regulators.

Introduction

Haploidization, protoplast fusion, gene transfer and exploitation of somaclonal variation are examples of *in vitro* culture techniques with potential for crop improvement. An efficient regeneration protocol either by organogenesis or somatic embryogenesis is a major prerequisite for the application of gene transfer methods. Regeneration of peanut by organogenesis from various explants, leaves, cotyledons, cotyledonary node, hypocotyl, epicotyl, zygotic embryos, has been reported (McKently *et al*. 1990, Eapen and George 1993), however, the regeneration frequency was low and plants were rarely established. A number of recent reports describe somatic embryogenesis in peanut using a variety of different explants, including leaves (Baker and Wetzstein 1992, Chengalrayan *et al*. 1994), immature cotyledons (Ozias Akins 1989, Eapen *et al*. 1993), immature embryos (Hazra *et al*. 1989) mature and dry seeds (Baker *et al*. 1995), hypocotyl (Venkatachalam *et al*. 1997a), cotyledons with or without embryos, epicotyls, leaflets (Cucco and Jaume 2000) and mature epicotyl (Little *et al*. 2000).

Mutations are known to enhance the genetic variability of crop plants and the efforts are being made to improve the genetic make up of groundnut crop for higher yield, oil content and development of cultivars resistant to diseases and pest. The combination of tissue cultures with mutation induction techniques may be an effective way to crop improvement (Gao *et al*. 1992). However, experience in applying radiations or chemical mutagens to *in vitro* cultured plant material is limited and there are only few reports on successful selection of mutants after *in vitro* application of mutagens (Micke

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; EC - embryogenic callus; EMS - ethyl methane sulphonate; GR - γ-radiation; Gy - Gray; MS - Murashige and Skoog; NAA - naphthaleneacetic acid; SA - sodium azide.
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et al. 1990). Standardization of optimal doses for ionizing radiation and chemical mutagens to plant tissue culture and their response on *in vitro* mutation efficiency has been reported in many major crops such as tobacco (Hell 1983), rice (Gao *et al*. 1992), groundnut (Venkatachalam and Jayabalan 1996), sugarcane (Khan *et al*. 1999), potato (Al-Safadi *et al*. 2000) and *Chrysanthemum* (Mandal *et al*. 2000). Although γ-radiation and chemical mutagens have been used to induce mutations in callus culture of groundnut (Venkatachalam and Jayabalan 1997b), there are no reports on inducing mutations *in vitro* with embryogenic callus (EC) of groundnut. In this study, ECs of groundnut were subjected to both physical and chemical mutagenesis, evaluated for responses, growth rate, number of somatic embryos, plantlet regeneration and variations in the regenerated plants.

Materials and methods

Two groundnut (*Arachis hypogaea* L.) cultivars, Co-5 and Co-7, were used in the present investigation and seeds were obtained from Tamil Nadu Agricultural University, Coimbatore, India. They were immersed in distilled water with a drop of *Tween 80* for 5 min and surface sterilized with 0.1 % (m/v) aqueous mercuric chloride for 7 - 10 min followed by 5 rinses in sterile distilled water. Seeds were germinated on Murashige and Skoog (1962; MS) basal medium under dark and temperature of 24 ± 2 °C. Hypocotyl explants (0.5 - 1.0 cm) from 7-d-old seedlings were placed separately in culture tubes with EC induction medium consisted of MS salts, B5 vitamins (Gamborg *et al*. 1968), 40 g dm^{-3} sucrose, 20 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg dm^{-3} 6-benzylaminopurine (BAP). The pH of the medium was adjusted to 5.8, 0.5 % (m/v) agar was added, and autoclaved for 15 min at 121 °C. All cultures were incubated at 24 ± 2 °C under cool-white fluorescent tubes providing irradiance of 40 μmol m^{-2} s^{-1} during a 16-h photoperiod.

After 5 weeks on induction media, ECs were exposed to 10, 20, 30, 40 and 50 Gray (Gy) of γ-radiation (GR) in a gamma cell (^{60}Co source) installed at the Sugarcane Breeding Institute, Coimbatore, India. After irradiation, EC were transferred to fresh medium. For ethyl methane sulphonate (EMS) and sodium azide (SA) treatments**,** the appropriate amounts of EMS or SA were mixed separately in MS basal medium and dissolved thoroughly for each treatment. The pH of the medium was adjusted to 6.0 and 3.0 for EMS and SA, respectively prior to adding the mutagens. The solutions were filter-sterilized with sterile *Millipore* 0.45 μm membrane filter in the laminar air flow chamber. The EC were treated with 1, 2, 3, 4 and 5 mM EMS or SA for 30 min. The doses/concentrations of both physical and chemical mutagens were selected based on previous experiments conducted to estimate LD_{50}. The treated EC were thoroughly rinsed with sterile MS liquid medium (basal) to rinse out any excess mutagens and blotted dry on sterile tissue paper. Then untreated EC (controls) and treated EC were cultured on above mentioned induction medium for 2 - 3 weeks to assess the survival rate of ECs and then plated on maturation medium containing MS salts, 3 % (m/v) sucrose, 2.0 mg dm^{-3} BAP, 0.5 mg dm^{-3} 2,4-D, and 0.7 % agar. The observations were made on number of EC plated, number of EC showing matured embryos and percent of embryo maturation was calculated. After reaching the dicotyledonary stage, the embryos were transferred along with small clumps of EC to MS medium containing BAP 2.0 mg dm^{-3}, 0.25 mg dm^{-3} naphthaleneacetic acid (NAA), 2 % sucrose, 3 % (m/v) activated charcoal for germination and conversion into plantlets. Morphological variations of the embryos during the developmental stages were noted with reference to the control.

Fully germinated embryos were separated from EC and transferred to test tubes with 20 cm^3 of growth regulator free MS medium with 0.7 % agar. Cultures were incubated for 10 to 15 d and plantlets with well-developed roots were transferred to plastic cups containing autoclaved mixture of sand, red soil and manure (1:1:1). Each plantlet was covered with a polythene bag and the cups were maintained in controlled temperature and 60 % relative humidity. After hardening, well established plants (R0 plants) were transferred to the pots (40 × 45 cm), and grown to maturity under recommended agricultural practices. R0 plants produced normal flowers and viable seeds in pods.

Each treatment consisted of at least 40 explants and the experiment was repeated thrice. The data on survival rate of embryogenic calli, growth rate of EC, number of EC plated, percent of matured embryos, mean number of embryos, percent of germination and variations in somatic embryos during developmental stages were subjected to analysis of variance (*ANOVA*) and Duncan's New Multiple Range Test (DNMRT).

Results and discussion

The mutagens used in the present study influenced survival rate of EC, fresh mass of EC, number of somatic embryos per explants, and percentage of germination of somatic embryos in the both groundnut cultivars. The exposed/treated hypocotyl explants were cultured on embryo induction medium consists for 5 weeks (Fig. 1*A*). The survival rate of EC (Fig. 1*B*) after mutagenic treatments remained the same as of control up to 30 Gy

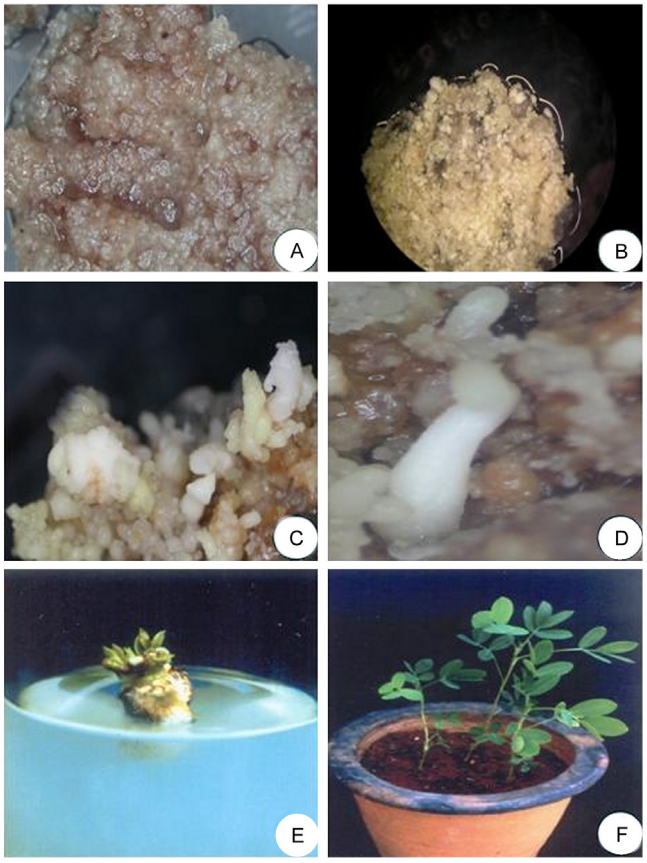

Fig. 1. Somatic embryogenesis in peanut and germination of plantlets from mutagen-treated embryognic callus: *A* - embryogenic callus after exposure to mutagens; *B* - induction of embryos from hypocotyl explant; *C* - differentiation of embryos on MS medium + 20 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} BAP; *D* - maturation of embryos on MS medium + 2.0 mg dm^{-3} BAP + 0.5 mg dm^{-3} 2,4-D; *E* - germination of embryos on MS medium + 2.0 mg dm^{-3} BAP + 0.25 mg dm^{-3} NAA; *F* - regenerated plants established in the pot.

Table 1. Effect of mutagens on survival rate and fresh mass of EC, number of EC plated, number of EC showing matured embryos and percent embryo maturation of groundnut. EC - embryogenic callus. Means within column with different letters are significant at 1 % level according to DNMRT.

Mutagens	Survival [%]		Fresh mass [g]		Number of EC plated		Number of embryos		Maturation [%]	
	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7
Control	100.0a	100.0a	0.500gh	0.515ij	20	25	11.2	14.1	56.6ij	60.4ij
GR 10 Gy	100.0a	100.0a	0.610cd	0.640c	30	25	20.8	18.1	69.3g	76.4f
GR 20 Gy	100.0a	100.0a	0.622c	0.681bc	28	34	22.2	28.4	79.3d	83.5d
GR 30 Gy	100.0a	100.0a	0.725a	0.775a	32	30	28.6	26.2	89.4a	87.3bc
GR 40 Gy	72.4b	78.5b	0.588ef	0.603de	32	26	18.7	16.4	58.4i	63.1i
GR 50 Gy	50.2d	54.6de	0.420hi	0.430l	28	24	15.6	12.2	55.7jk	50.8k
EMS 1 mM	100.0a	100.0a	0.520g	0.540fg	36	30	28.4	25.6	77.7e	83.3de
EMS 2 mM	100.0a	100.0a	0.543f	0.578e	38	34	32.6	29.5	84.2bc	85.2cd
EMS 3 mM	100.0a	100.0a	0.664b	0.688b	34	30	30.2	26.1	88.2ab	86.6c
EMS 4 mM	70.2bc	72.6bc	0.432h	0.499k	30	25	22.4	18.4	73.3f	72.0g
EMS 5 mM	48.8de	58.9d	0.390ij	0.400m	27	25	18.6	17.3	66.6h	68.0h
SA 1 mM	100.0a	100.0a	0.520g	0.529h	28	32	22.8	28.4	78.6de	87.5b
SA 2 mM	100.0a	100.0a	0.534fg	0.549f	31	34	26.1	28.6	83.2c	82.4e
SA 3 mM	100.0a	100.0a	0.600e	0.612d	35	32	30.6	29.3	85.7b	90.6a
SA 4 mM	60.4c	68.5c	0.510gh	0.515i	30	27	22.4	19.4	73.3f	70.3gh
SA 5 mM	43.2f	50.4f	0.410i	0.422lm	25	24	14.6	15.4	56.0j	62.5ij

Table 2. Effect of mutagens on mean number of somatic embryos per EC, germination percentage of embryos and morphological variations during the developmental stages of somatic embryos in groundnut. EC-embryogenic callus. Means within column with different letters are significant at 1 % level. The numbers within the parenthesis showed the frequency of morphological variations of embryos.

Mutagens	Number of embryos		Germination of embryos [%]		Morphological variations heart		torpedo		cotyledonary	
	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7
Control	4.0e	5.0e	48.2h	50.7h	-	-	-	-	-	-
GR 10 Gy	5.0d	8.0b	52.1g	55.2g	-	-	-	1 (3.3)	-	-
GR 20 Gy	6.0c	8.0b	61.2e	65.2de	2 (6.6)	1 (3.3)	1 (3.3)	2 (6.6)	3 (10.0)	2 (6.6)
GR 30 Gy	8.0a	9.0a	70.7a	78.6a	2 (6.6)	2 (6.6)	2 (6.6)	2 (6.6)	3 (10.0)	3 (10.0)
GR 40 Gy	6.0c	7.0c	58.2f	62.4e	4 (13.3)	3 (10.0)	2 (6.6)	6 (20.0)	4 (13.3)	5 (16.6)
GR 50 Gy	4.0e	4.0f	40.6j	45.2i	5 (16.6)	5 (16.6)	4 (13.3)	6 (20.0)	6 (20.0)	8 (26.6)
EMS 1 mM	5.0d	5.0e	61.6d	60.7f	-	-	-	1 (3.3)	-	-
EMS 2 mM	6.0c	6.0d	64.2c	68.3c	1 (3.3)	2 (6.6)	2 (6.6)	2 (6.6)	4 (13.3)	2 (6.6)
EMS 3 mM	7.0b	8.0b	69.7ab	70.3bc	2 (6.6)	2 (6.6)	2 (6.6)	4 (13.3)	4 (13.3)	4 (13.3)
EMS 4 mM	5.0d	5.0e	56.4fg	60.2fg	2 (6.6)	4 (13.3)	3 (10.0)	4 (13.3)	4 (13.3)	6 (20.0)
EMS 5 mM	4.0e	4.0f	40.2i	44.6ij	5 (16.6)	6 (20.0)	4 (13.3)	6 (20.0)	7. (23.3)	8 (26.6)
SA 1 mM	6.0c	5.0e	58.2f	60.2fg	-	-	2 (6.6)	2 (6.6)	-	-
SA 2 mM	6.0c	6.0d	61.4de	65.5d	2 (6.6)	1 (3.3)	2 (6.6)	3 (10.0)	4 (13.3)	2 (6.6)
SA 3 mM	7.0b	7.0c	68.7b	72.2b	2 (6.6)	2 (6.6)	4 (13.3)	4 (13.3)	4 (13.3)	4 (13.3)
SA 4 mM	5.0d	5.0e	56.4fg	62.2ef	4 (13.3)	2 (6.6)	5 (16.6)	5 (16.6)	5 (16.6)	6 (16.6)
SA 5 mM	4.0e	4.0f	38.2k	43.6j	6 (20.0)	5 (16.6)	5 (16.6)	6 (20.0)	8 (26.6)	8 (20.0)

Table 3. Effect of mutagens on morphological and yield characters (mean values) of groundnut (R_0 plants). Means within column with different letters are significant at 1 % level.

Mutagens	Plant height [cm]		Days to flowering		Number of branches		Number of pods		Pod yield [g]	
	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7	Co. 5	Co. 7
Control	35.8h	36.4e	29.5f	28.5g	10.4g	12.5fg	30.7g	32.6f	17.6gh	17.8gh
GR 10 Gy	37.2fg	37.4d	29.8ef	28.4gh	11.2f	12.6f	34.6e	35.2cd	20.4ef	20.7de
GR 20 Gy	38.5cd	39.4bc	27.4i	25.4k	12.3d	13.8cd	38.4bc	36.4bc	22.7b	24.6a
GR 30 Gy	45.2a	40.4a	30.2e	34.1ef	15.3a	16.8a	40.2a	39.2a	24.7a	20.8d
GR 40 Gy	36.4gh	36.1ef	32.3de	34.2e	9.3i	8.4k	24.7h	26.2gh	16.5j	16.8i
GR 50 Gy	32.4i	30.3i	35.8b	36.4b	8.9j	9.1ij	21.5jk	21.7ij	17.4i	17.5h
EMS 1 mM	38.2de	37.2de	28.3h	27.2i	10.8fg	12.8ef	33.8ef	34.2h	18.4h	19.4f
EMS 2 mM	38.8c	39.8b	26.4jk	26.4j	12.0de	13.0e	37.2cd	35.4cd	21.4cd	21.8bc
EMS 3 mM	43.4b	40.3ab	26.5j	26.4j	14.6b	15.2b	39.2b	36.4c	21.6c	22.3b
EMS 4 mM	38.4d	39.4bc	32.4d	35.4c	9.0ij	8.2kl	22.7j	25.4h	18.4h	16.4j
EMS 5 mM	30.3j	34.3g	36.9a	38.7ab	8.6jk	9.0j	20.7kl	20.6i	17.2i	14.3k
SA 1 mM	36.4gh	36.4e	29.2fg	28.2h	10.0gh	11.4h	32.8f	34.3hi	18.0hi	18.4g
SA 2 mM	37.5e	38.8cd	26.4jk	27.2i	11.8e	12.6f	37.6c	35.4g	19.2g	17.3hi
SA 3 mM	42.5bc	38.9c	26.1k	26.4j	13.7c	14.0c	39.8ab	36.7cd	21.2cd	20.6de
SA 4 mM	37.4ef	35.5f	34.4c	35.2cd	8.1l	9.2i	24.3hi	22.7e	20.6e	16.5ij
SA 5 mM	30.1jk	31.2h	36.6ab	38.8a	7.6m	8.4k	21.4k	26.4jk	16.4jk	12.8l

and 3 mM concentrations whereas they showed decreased trends over control at higher doses/concentrations (40 or 50 Gy GR and 4 or 5 mM EMS and SA). Lowest survival rate was noted at 5 mM SA (Table 1). With increasing dose of GR, similar survival rate of callus cultures in *Citrus* (Srivastava *et al*. 2001) and carrot (Pawlicki *et al*. 2001) was reported but decreased survival rate of embryogenic cultures in banana (Kulkarni *et al*. 2004) and rice (Kim *et al*. 2004a).

An increase in EC fresh mass was observed upto 30 Gy/3 mM after that fresh mass decreased. The maximum fresh mass of EC (0.775 g) was noted at 30 Gy GR followed by EMS and SA in both cultivars (Table 1). Previously, Muthusamy (2001) observed an increase in growth rate of ovular callus of cotton with lower concentrations of mutagens. Palanivel (1998) subjected different explants of groundnut to different concentrations of mutagens including GR, EMS and SA and also found that the lower dose/concentrations of mutagens increased growth rate of callus, multiple shoot induction, somatic embryo formation and germination of embryos. Lukey (1982) observed that low doses of ionizing

radiations have stimulatory effect on plant growth. Pawlicki *et al*. (2001) observed that the callogenesis was increased at lower concentration of mutagens (5, 10 Gy of GR). In potato (Ahloowalia 1990) and *Setaria italica* (Reddy and Vaidyanath 1990) lower mutagen dose/ concentration stimulated their growth while higher doses inhibited their growth.

At the end of third week, the embryos were observed visually (Fig. 1*C*) and the EC was plated on maturation medium. The mean number of somatic embryos per explant increased at lower doses/concentrations of mutagens relative to control (Fig. 1*D*). The maximum number of somatic embryos (9.0 per explant) was noted at 30 Gy GR treatments in both cultivars followed by 3 mM EMS and 3 mM SA.

The somatic embryos germinated into whole plants (38.2 - 78.6 %) following standard protocols of induction, maturation and regeneration for groundnut (Venkatachalam *et al*. 1997a). The percentage of germination was higher at GR upto 30 Gy and EMS and SA upto 3 mM than at GR 40 Gy or 4 and 5 mM EMS or SA (Fig. 1*E*, Table 2). Germinated embryos were separated from the EC and transferred onto hormone free MS medium for further growth of shoots and roots. After 10-15 days, plants with well-developed roots were transferred to pots containing a 1:1:1 mixture of sand, red soil and manure (Fig. 1*F*). To our knowledge, this is the first study whereby EC of groundnut were subjected to *in vitro* mutagenesis, leading to successful development of somatic embryos and whole plantlets.

Mutagenic treatments followed by tissue culture of explants enhanced the number of shoots regenerated from each explant at the lower doses, whereas it decreased proportionally with increase of dosage (Swanson *et al*. 1989). Similar observations were reported in *Gerbera* (Laneri *et al*. 1990), *Asparagus* (Delbreil and Jullien 1994), groundnut (Venkatachalam *et al*. 1999, Venkatachalam 2000), cassava (Lee *et al*. 1997, Joseph *et al*. 1999b, Roy *et al*. 2004), *Rosa* (Ibrahim *et al*. 1998), mango (Mazanilla-Ramirez *et al*. 2001), *Gladiolus* (Kasumi *et al*. 2001), sweet potato (Lee *et al*. 2002), lotus (Arunyanart and Soontronyatara 2002) and rice (Lee *et al*. 2003).

During the embryo and plantlet development different morphological abnormalities were observed (Table 2). There were no variations in controls, 10 Gy GR and 1 mM EMS and 1 mM SA treatments. The number and frequency of the abnormalities was increased with increasing doses/concentrations of mutagens. The number of abnormalities was higher in cotyledonary stage than in early stages (Table 2). Our results are similar to variations in somatic embryos induced by GR recently reported by Lee *et al*. (2002) and Roy *et al*. (2004). The number of leaf variations also increased with increasing concentrations of mutagens (data not shown). The present study clearly shows that there were a large number of embryo abnormalities and leaf variations during development of embryos to complete plantlets. The results obtained with leaf variations were similar to those previously reported for shoot tip culture with *in vitro* mutagenesis in cotton (Muthusamy and Jayabalan 2000, 2002), carrot (Pawlicki *et al*. 2001), *Citrus* (Srivastava *et al*. 2001) and potato (Das *et al*. 2001).

A range of morphological variations were also observed from 1-month-old R0 plants to maturity (Table 3). Five important characteristics (plant height, days to flowering, number of branches, number of mature pods and pod yield) were selected to analyze the influence of mutagenic treatments on performance of plants. The lower doses/concentrations of mutagens increased the morphological and yield parameters over those in controls whereas the decreased trend was noted at higher doses/concentrations. The changes observed in different R0 plants may be due to genetic variations caused by mutagenic treatments. Some of the R0 plants obtained from these studies were promising with respect to selected characters. Further experiments are necessary to study future generations from the R0 seed for the stability of the induced characters.

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