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RESEARCH ARTICLE

EFFECT OF IMIDACLOPRID SEED TREATMENT ON THE DEFENSE SYSTEM OF *BACILLUS THURINGIENSIS* COTTON SEEDLINGS

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ABSTRACT

Bacillus thuringiensis cotton seeds are treated with insecticides for protection against pathogenic organism's infestation. The use of insecticides as seed treatments entails both economic benefits and potential risks like phytotoxicity. The present study was carried out to evaluate the biochemical effect of majorly used neonicotinoid seed treatment insecticide imidacloprid (trade name Gaucho 70 WS) at recommended concentration (3.2g/Kg seeds) on the growth of seedlings (length, biomass) of three Bt cotton hybrids (RCH-134, JKCH-1947, NCEH-6R), by analyzing their defense related parameters on third and eighth day after sowing of treated seeds under controlled conditions. It was observed that imidacloprid increased the activity of defense enzyme peroxidase significantly w.r.t controls (at $p<0.01$) on 8th day after germination and non significantly on 3rd day in both RCH-134 and JKCH-1947 hybrids. Superoxide dismutase activity showed significant increase (at $p<0.01$) from 1.71±0.53 ΔE/min/mg protein to 6.72±1.23 ΔE/min/mg protein in NCEH-6R plumules and at $p<0.05$ in JKCH-1947 plumules from 0.71±0.13 ΔE/min/mg protein to 4.95±0.52 ΔE/min/mg protein. A non significant increase was found in Phenylalanine ammonia lyase activity in all three hybrid seedlings . The levels of *Bacillus thuringiensis* protein increased significantly (at $p<0.01$) in RCH-134 and NCEH-6R seedlings on 3rd day after germination and (at $p<0.05$) on 8th day after germination. Total phenols were also increased significantly (at $p<0.01$) with respect to control seedlings in all three Bt cotton hybrid seedlings on both days of observation. Length and biomass of seedlings showed no significant and variable increase. The results suggested that the seed treatment with imidacloprid enhanced the defense system of the seedlings and might help to increase the cotton crop quality and productivity.

INTRODUCTION

Cotton plays a vital role in economic growth of India. Due to rising demand of cotton industry, the Indian cotton cultivation sector has been increasing its quality and productivity by cultivation of transgenic Bt cotton varieties, plant protection technologies and encouraging use of quality seeds and insecticides. Transgenic cotton is genetically modified to contain a Bt gene derived from soil bacterium *Bacillus thuringiensis* which results in expression of Bt protein/Cry protein which is toxic and provide effective control of *Helicoverpa armigera*, the most destructive lepidopteran pests on cotton. Insecticidal efficacy of Bt cotton is dependent upon Bt protein content in plant parts (Luo *et al*., 2008). So Bt toxin protein act as a defense protein in Bt cotton crop. Along with the Bt protein, other important components which comprise the defense system of the plant include enzymes, peroxidase, superoxide dismutase, phenylalanine ammonia lyase (Verma and Dubey, 2003, Nakkeran *et al*., 2006) and secondary metabolites such as total phenols (Daniel *et al*., 1999). Defense system of the growing seedling is of prime importance during the early phases of plant life. Peroxidase and superoxide dismutase are the crucial enzymes involved during germination and early seedling growth (Pergo and Ishii-Iwamoto, 2011). So the above parameters are of great significance during germination and early stages of cotton plant life cycle, but these defence related compounds are influenced by various external factors such as water logging, salinity ,water deficit and temperature (Martins *et al*., 2008, Gonias *et al*., 2008) The extensive use of insecticides can be the other major external factor to effect the components of defense system . Imidacloprid is the majorly used cotton seed treatment insecticide which provides additional protection against infestation by pathogenic organisms to ensure proper germination. However our knowledge of the response of transgenic Bt cotton seedlings to insecticide seed treatment is

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still limited, especially for the effect on Bt protein which is an important biochemical parameter to ensure high yield. Seed treatment with insecticides exerts both phytotoxic and stimulatory influences on plant germination and growth by interfering with enzyme pathways (Hanley and Whiting, 2005). Our previous work showed that the foliar spray of imidacloprid on Bt cotton plants enhanced the overall plant health and growth (Kaur *et al*., 2011). In present study, we investigated the effect of imidacloprid seed treatment on germination and health of growing seedling by analyzing the Bt toxin content, defense related enzymes (peroxidase, superoxide dismutase, phenylalanine ammonia lyase) and secondary metabolites (total phenols) along with physiological parameters such as length and fresh weight of three Bt cotton hybrid seedlings (RCH-134, JKCH-1947, NCEH-6R).

MATERIALS AND METHODS

The present study was conducted in the Department of Biochemistry, Punjab Agricultural University (PAU), Ludhiana, Punjab (India) with three replications of RCH-134, NCEH-6R and JKCH-1947 hybrids. RCH-134 hybrid contain Bt gene developed by Monsanto, USA, JKCH-1947 has modified Cry1Ac gene construct developed by Indian Institute of Technology Kharagpur while NCEH-6R contain fused genes Cry1Ac and Cry1Ab developed by Chinese Academy of Agricutural Sciences.

Plant Material

Untreated seeds of three *Gossypium hirusutam* Bt hybrids i.e RCH-134, NCEH-6R and JKCH-1947 were procured from Rasi seeds (P) Ltd Tamilnadu, Nath Biogene Limited Aurangabad and JK Agrigenetics Ltd Hyderabad, respectively. Insecticide imidacloprid (tradename Gaucho 70 WS, 700g Imidacloprid/Kg) were bought from Bayer Limited.

Insecticide treatment

Forty seeds were weighed and soaked in 4.5 ml of water (the quantity of water measured i.e completely absorbed by forty seeds overnight) with imidacloprid at dose levels of 3.2 g/Kg of seeds for overnight. The treated (imidacloprid) and untreated (control) seeds were germinated in small pots (10 seeds/pot) containing field soil in seed germinator under 16/8 hr light/dark period at 28±2 degree celsius. Three pots were used for each hybrid and labeled accordingly. Pots were watered daily throughout the experiment.

Sample Collection

Cotyledon, plumule and radicle of the seedling were collected on third and eighth day after sowing (DAS) to determine the expression level of Bt toxin and activity of peroxidase, superoxide dismutase and phenylalanine ammonia lyase on fresh samples. Total phenol level was estimated using dry samples (dried at 70 degree Celsius) of cotyledon, plumule and radicle.

Bacillus thuringiensis (Bt) toxin quantification

The level of Cry toxin in cotyledons was quantified on third and eighth day after sowing. It was determined by commercial 96-well microplate format sandwich ELISAs, EnviroLogix Cry1Ab/Cry1Ac QuantiPlate® (AP003) as described by Szekacs *et al*. (2010). Cotyledon discs were weighed and homogenized in extraction solution provided with the ELISA kit. Solids were allowed to settle for few minutes and supernatant was used to perform ELISA as per manufactures protocol. Quantification of Cry1Ac was done by plotting absorbance values of the test samples on the standard curve generated with purified Cry1Ac standards on each of the ELISA plates and expressed as µg Cry1Ac per g fresh weight of the tissue.

Peroxidase Activity (POD) EC 1.11.1.7

The enzyme POD was extracted and its activity was estimated by the method of Shannon *et al*. (1966) with 0.1 M sodium phosphate buffer (pH 7.5). Rate of change of absorbance was recorded at 470 nm after every 30 s up to 3 min resulting from guaicol oxidation to tetraguaicol in the presence of hydrogen peroxide.

Superoxide dismutase Activity (SOD) EC 1.15.1.1

The activity of SOD enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.5) was estimated by the method of Marklund and Marklund (1974) by observing the rate of autooxidation of pyrogallol at 420 nm after a lap of 30 sec upto 3 minutes.

Phenylalanine ammonia lyase Activity (PAL) EC 4.3.1.24

PAL enzyme was extracted and the activity was determined as described by Burrell and Rees (1974) by following the appearance of t-cinnamic acid resulting from the deamination of L-phenylalanine.

Total phenols

Samples OF cotyledon, plumule and radicle were dried, weighed and total phenols were extracted by refluxing the dry sample material with 80% methanol and total phenols obtained were estimated by using the method of Swain and Hillis (Swain and Hillis, 1959).

Seedling growth

Length and fresh weight of plumule and radicle of 5 days old seedlings was measured Three seedlings in each pot were taken and an average of three pots was calculated for each hybrid.

Statistical analysis

The data was statistically analyzed using Student's-t test. The difference from the control and treated group was considered significant at $p \leq 0.01$ and $p \leq 0.05$. All the results were expressed as Mean ± Standard Deviation (SD).

RESULTS

Bt expression

Significant increase in Bt toxin level of cotyledons was observed in RCH-134 (at $p \leq 0.01$) and NCEH-6R (at $p \leq 0.05$) treated hybrids as compared to their respective controls on third and eighth day after sowing. However the increase was non significant with 4.68% and 6.69% in JKCH-1947 treated hybrid cotton seedlings on both days of observation respectively (Table 1). Overall the intervarietal variations are observed in the increase of Bt toxin level after imidacloprid treatment. Bt toxin level was almost four times higher in NCEH-6R and JKCH-1947 control hybrids than RCH-134 control hybrid.

Peroxidase Activity

After imidacloprid treatment the activity of peroxidase in cotyledons of RCH-134 and JKCH-1947 hybrid seedlings was found to be increased non significantly as compared to their

controls on third day after sowing (Table 2) and the activity was decreased non significantly by 18.81% in cotyledons of NCEH-6R hybrid seedlings. Treated seedlings of all three hybrids showed non significant increase on third day in peroxidase activity of plumule and radicle. On eighth day, the enzyme activity was increased significantly from 6.43±1.45 ΔE/min/mg protein to 17.93±2.56 ΔE/min/mg protein in RCH-134 cotyledons and from 7.92±1.34 ΔE/min/mg protein to 12.81±2.86 ΔE/min/mg protein in NCEH-6R cotyledons respectively. The increase was non significant in JKCH-1947 hybrid cotyledons on eighth day after sowing. Plumule and radicle of eight days old seedlings of RCH-134 and JKCH-1947 hybrids showed significant increase in POD activity but the increase was non significant in plumule and radicle of NCEH-6R seedlings. In general the increase in POD enzyme activity was more and significant on eighth day after treatment in all hybrid seedlings as compared to their respective controls.

Table 1 Effect of soaking seeds with imidacloprid on the Bt-toxin level (µg/gm F.W) in cotyledons of germinating Bt cotton seedlings.

	3rd Day after Sowing (DAS)		
	RCH-134	**NCEH-6**	JKCH-1947
Control	6.21 ± 0.92[a]	33.65 ± 0.53	25.83 ± 2.33
Imidacloprid*	9.32 ± 0.61 (+50.00%)[b**]	38.51 ± 0.82 (+14.44%)**	27.04 ± 3.82 (+4.68%)
	8th day after Sowing (DAS)		
Control	7.83 ± 0.73	35.12 ± 1.31	28.53 ± 1.75
Imidacloprid*	9.24 ± 0.24 (+18.00%)*	38.41 ± 1.42 (+9.36%)*	30.44 ± 1.53 (+6.69%)

[a] Mean±SD of three replicates
[b] Values in parenthesis represent percentage change w.r.t control
* $p \leq 0.05$, ** $p \leq 0.01$

Superoxide dismutase Activity

Cotyledons of treated seedlings of RCH-134 hybrid showed non significant increase in superoxide dismutase activity on third day after sowing and significant increase (at $p \leq 0.05$) from 0.84±0.12 unit enzyme/mg protein to 2.24±0.63 unit enzyme/mg protein on eighth day. The increase was non significant in cotyledons of other two hybrids on both third and eighth day after sowing as compared to their controls (Table 2) except the levels of SOD were almost same to control in three day old NCEH-6R seedlings. The SOD activity in plumule of treated RCH-134 seedlings was increased significantly by 41.41% on third day and almost no change on eighth day after sowing w.r.t controls. NCEH-6R plumule showed significant increase at $p \leq 0.01$ on both third and eighth day. The increase was non significant on third and significant on eighth day in JKCH-1947 plumules. The enzyme activity in radicle of RCH-134 hybrid was observed to be increased non significantly by 11.94% and significantly by 54.61% on third and eighth day respectively after sowing in comparison to their controls. Radicles of seedlings had significant increase at $p \leq 0.05$ and non significant increase in NCEH-6R and JKCH-1947 respectively on both days of observation. The levels of SOD were raised more in plumule and radicle than the cotyledons in treated seedlings.

Phenylalanine Ammonia lyase Activity

The results of PAL level were summarized in table 3. A nominal increase was observed in PAL activity of cotyledons of all three hybrids on third as well eighth day, with more increase on eighth day w.r.t control seedlings. Activity in plumule of RCH-134 hybrid increased non significantly by 31.57% and 17.80% on third and eighth day respectively after sowing. Plumule of NCEH-6R and JKCH-1947 treated hybrids also showed non significant increase in PAL activity. Similarly radicle of all three hybrids had minor increase in PAL activity after imidacloprid treatment.

Total Phenols

Significantly higher level of total phenols in cotyledons and radicle was observed (at $p \leq 0.01$) in all treated hybrids of Bt cotton seedlings from their respective controls on both days of observation. Value of total phenols in plumule of RCH-134 hybrid seedlings was significantly increased (at $p \leq 0.01$) from 9.01±1.02 mg/g dry weight to 18.43±2.21 mg/g dry weight after treatment with imidacloprid on third day and non significantly higher value on eighth day w.r.t their controls. Plumule of other two hybrids also showed significant increase on both days as compared to their controls. (Table 3)

Length and fresh weight of seedlings

The plumule length of treated seedlings was increased non significantly in RCH-134 and NCEH-6R hybrids and significantly in JKCH-1947 seedlings from 13.21±0.91 cm to 15.93±0.54 cm (Table 4). In case of radicle length, their was significant increase by 47.28% and 34.55% in RCH-134 and JKCH-1947 treated hybrids as compared to their control ones.

The imidacloprid treatment showed non significant increase in NCEH-6R hybrid seedlings.

DISCUSSION

Bt protein is a toxic protein which provide defense to Bt cotton plants against different orders of insects especially lepidopterans. This study showed that the treatment of seeds with imidacloprid increased the expression of Bt protein in all the three hybrid seedlings RCH-134, NCEH-6R, JKCH-1947 (Table1). Our previous work reports that foliar treatment of imidacloprid also results in increased levels of Bt toxin in Bt cotton plants (Kaur *et al*., 2011). Under imidacloprid treatment the rate of photosynthesis is high (Gonias *et al*., 2008) in treated seedlings which could increase the protein production and this may result in high Bt protein levels. Apart from Bt protein the defense system of transgenic plants include two important antioxidant enzymes, peroxidase and superoxide dismutase. Peroxidase is an enzyme involved in removal of H_2O_2, oxidation of toxic reductants, polymerization of phenolic monomers and in lignifications process that adds strength to the plant cell wall during growth (Heldt, 1997). Superoxide dismutase maintain the steady level of reactive oxygen species (Ananieva *et al*., 2004) produced from various physiological and redox reactions in the cells. In present study we observed that overall the activity of peroxidase was increased non significantly on third and significantly on eighth day after sowing in RCH-134 and JKCH-1947 hybrid seedlings while the increase was non significant in case of NCEH-6R seedlings. Activity of SOD enzyme also increased in all the parts of treated hybrid seedlings, with more significant increase in plumule and radicle of NCEH-6R hybrid as compared to other hybrids. Thus imidacloprid enhanced the antioxidant defense system of the cells.

Table 2 Effect of soaking seeds with imidacloprid on peroxidase (ΔE/min/mg protein) and superoxide dismutase activity (unit enzyme/mg protein) in Bt cotton germinating seedlings

Germinating seedling	Treatment	RCH-134				NCEH-6R				JKCH-1947			
		3 DAS		8 DAS		3 DAS		8 DAS		3 DAS		8 DAS	
		POD	SOD	POD	SOD	POD	SOD	POD	SOD	POD	SOD	POD	SOD
Cotyledon	Control	8.52±1.34[a]	1.54±0.22	6.43±1.45	0.84±0.12	7.92±1.34	2.22±0.23	7.92±1.34	1.71±0.53	3.80±0.72	1.11±0.23	6.52±1.41	0.71±0.13
	Treated	8.86±2.58 (+3.99%)[b]	1.81±0.13 (+17.53%)	17.93±2.56 (+178.84%)**	2.24±0.63 (+166.66%)*	6.43±1.56 (-18.81%)	2.21±0.13 (-0.45%)	12.81±2.86 (+61.74%)*	1.95±0.74 (+14.03%)	3.92±0.95 (+3.16%)	1.92±0.22 (+72.97%)	9.26±3.43 (+42.02%)	0.80±0.22 (+12.67%)
Plumule	Control	2.24±0.75	5.53±0.15	2.94±0.37	2.91±0.42	1.83±0.55	5.62±1.13	4.85±0.66	2.32±0.91	2.43±0.82	1.72±0.34	2.92±0.66	3.92±0.43
	Treated	2.82±0.56 (+25.89%)	7.82±0.21 (+41.41%)**	7.92±0.31 (+169.38%) **	2.96±0.36 (+1.72%)	1.92±0.16 (+4.91%)	7.94±0.46 (+41.28%)**	6.41±0.74 (+32.16%)	6.72±1.23 (+65.47%)**	2.61±0.55 (+7.40%)	2.23±0.45 (+29.65%)	8.95±1.23 (+206.50%)**	4.95±0.52 (+26.27%)*
Radicle	Control	3.34±1.33	2.01±0.44	3.84±0.74	1.52±0.21	2.35±0.72	2.03±0.36	5.15±1.06	2.02±0.34	2.12±0.25	2.13±0.14	1.82±0.26	2.52±0.31
	Treated	3.63±0.86 (+8.68%)	2.25±0.16 (+11.94%)	10.32±1.91 (+168.75%)**	2.35±0.16 (+54.61%)**	2.91±0.20 (+23.82%)	3.04±0.23 (+49.75%)*	7.43±1.31 (+34.84%)	3.92±0.55 (+94.05%)*	2.63±0.44 (+24.05%)	2.42±0.21 (+13.61%)	9.91±1.64 (+444.50%)**	3.12±0.44 (+23.80%)

[a] Mean ± S.D. of three replicates
[b] Values in parenthesis represent percentage change w.r.t control.
*$p \leq 0.05$
**$p \leq 0.01$

Table 3 Effect of soaking seeds with imidacloprid on Phenylalanine ammonia lyase activity (ΔE/min/mg protein) and total phenols (mg/g dry weight) in Bt cotton germinating seedlings

Germinating seedling	Treatment	RCH-134				NCEH-6R				JKCH-1947			
		3 DAS		8 DAS		3 DAS		8 DAS		3 DAS		8 DAS	
		PAL	Total Phenols	PAL	Total Phenols	PAL	Total Phenols	PAL	Total Phenols	PAL	Total Phenols	PAL	Total Phenols
Cotyledon	Control	1.11±0.21	10.11±1.52	1.13±0.74	9.12±1.83	0.74±0.23	11.22±0.51	0.53±0.14	10.03±1.14	0.92±0.23	12.13±2.15	0.72±0.23	15.53±0.25
	Treated	1.42±0.03 (+27.92%)	20.44±2.12 (+102.17) **	2.02±0.51 (+78.76%)	22.04±1.52 (+141.66%)**	0.82±0.14 (+10.81%)	24.51±2.22 (118.44%)**	0.72±0.34 (+35.84%)	20.23±0.34 (+101.69%)**	1.11±0.12 (+20.65%)	26.82±3.35 (+121.10%)**	0.91±0.31 (+26.38%)	27.32±0.16 (+75.91%)**
Plumule	Control	1.33±0.72	9.01±1.02	2.64±0.90	7.62±3.64	2.05±0.34	8.31±1.74	3.01±0.51	10.43±0.96	2.53±0.55	11.82±1.01	2.76±0.47	10.52±0.77
	Treated	1.75±0.34 (+31.57%)	18.43±2.21 (+104.55%)**	3.11±1.00 (+17.80%)	11.82±0.71 (+55.11%)	2.11±0.24 (+2.92%)	19.93±1.22 (+139.83%)**	3.32±0.81 (+10.29%)	14.33±0.32 (+37.39%)**	2.82±0.31 (+11.46%)	23.48±1.39 (+98.64%)**	2.93±1.22 (+6.15%)	15.13±2.58 (+43.82%)*
Radicle	Control	2.35±0.15	8.84±0.97	3.23±0.62	8.33±1.92	1.33±0.32	9.12±1.81	2.93±0.13	9.32±1.34	2.63±0.34	10.22±0.85	3.61±0.83	8.83±1.06
	Treated	2.53±0.22 (+7.65%)	17.92±1.33 (+102.71%)**	4.04±0.82 (+25.07%)	20.55±2.14 (+146.69%)**	1.81±0.52 (+36.09%)	19.54±2.83 (+114.25%)**	3.04±0.62 (+3.75%)	19.34±2.66 (+107.51%)**	3.33±0.33 (+26.61%)	24.83±2.54 (+142.95%)**	4.44±1.63 (+22.99%)	16.83±1.85 (+90.60%)**

[a] Mean ± S.D. of three replicates
[b] Values in parenthesis represent percentage change w.r.t control.
*$p \leq 0.05$
**$p \leq 0.01$

Table 4 Effect of soaking seeds with imidacloprid on Length (cm) and Fresh weight (mg) of plumule and radicle in Bt cotton seedlings

	Length (cm)					
	Plumule			Radicle		
	RCH-134	**NCEH-6R**	**JKCH-1947**	**RCH-134**	**NCEH-6R**	JKCH-1947
Control	14.13 ± 1.15[a]	13.91±0.82	13.21±0.91	4.42±0.63	5.41±0.42	4.63±0.32
Imidacloprid	14.86±0.27 (+5.16%)[b]	15.34±0.76 (+10.28%)	14.93±0.54 (+13.02%)*	6.51±0.60 (+47.28%)**	5.93±0.94 (+9.61%)	6.23±0.14 (+34.55%)**
	Fresh weight (mg)					
Control	161.14±9.20[a]	152.33±5.53	136.14±7.85	20.11±1.52	16.33±4.12	18.23±3.21
Imidacloprid	171.03±7.14 (+6.13%)[b]	155.21±7.71 (+1.89%)	180.37±6.18 (+32.48%)**	27.13±2.24 (+34.90%)*	20.64±2.15 (+26.39%)	19.96±4.47 (+9.48%)

Reactive oxygen species (ROS) such as superoxide radicles (O_2^-) and H_2O_2 are produced as secondary products of respiration by mitochondria or lipid degradation by glyoxysomes during germination and early seedling growth (Bailly, 2004) and their scavenging occurs through POD and SOD enzymes (Bowler *et al*., 1991). Increased levels of POD and SOD reduce oxidative damage to cell structures which occurs through increased production of ROS. Post application of imidacloprid seed treatment results in increased levels of PAL enzyme (non significantly) and total phenols (significantly) in all Bt cotton hybrid seedlings (Table 3). PAL is the entry enzyme into the phenylpropanoid pathway that is responsible for the synthesis of diverse array of phenolic metabolites such as caffeic, p-coumaric, sinapic acid, flavonoids, tannins and the structural polymer lignin. Phenolics play a significant role in plant protection against various stresses, in the regulation of plant metabolic processes and overall plant growth, as well as lignin synthesis (Jones, 1984).

Phenolic synthesis is regulated through the alternative proline-linked pentose phosphate pathway (Kalidas, 2004). So the stimulation of pentose phosphate pathway by imidacloprid allows the overexpression and significant increase in phenolics. This is in agreement with the study of (Horii *et al*., 2007) which also reported increased levels of phenolic content in corn seedlings by treatment of insecticide thiamethoxam which has been linked to higher pentose phosphate pathway activity. The length of treated seedlings was increased variably in plumule and radicle of all the three hybrids. Imidacloprid treatment might have positive effect on seed vigour during germination of cotton. It is hypotheisized that the apparent growth advantage seen in increased length and fresh weight of seedlings, by imidacloprid was due to the physiological and biochemical changes which includes activation of antioxidant enzymes and increase in phenolic content.

CONCLUSION

It can be concluded that imidacloprid might have both insecticidal and plant growth regulatory properties which induce morphological and biochemical changes. The results suggested that treating seeds with imidacloprid had significant implications through the improved seed vigour as reflected in antioxidant, phenolic and physiological linked responses. Enhancing seed vigour has the potential to improve overall plant productivity.

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