Survival and nodulation of *Bradyrhizobium japonicum* strain CJ02 on soybean (*Glycine max* (L.) Merrill) (cv. Cujut) cultivated on ferrasols (in pots experiment)

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Abstract: - A pot-experiment was conducted to study the effect of carboxyl methyl-cellulose (CMC), ash (by-product from cooking) and organic matter (compost) on survival of *Bradyrhizobium japonicum* strain CJ02, growth and yield of soybean (cv. Cujut) cultivated on ferrasols. Results indicated that CMC, ash and organic matter affected to the survival of bradyrhizobia and good nodulation (number of nodule and dry weight of nodule) significantly. Besides, the increases in plant height, shoot dry weight, pod dry weight, yield, and quality of soybean seed were also recorded as comparing with the control and *Bradyrhizobium* inoculation. However, *Bradyrhizobium* inoculation significantly increased plant height, shoot dry weight and pod dry weight in comparison with the control. Application of *Bradyrhizobium* [with CMC] inoculation plus ash and organic matter could be a useful practice, sustaining the growth and yield of soybean cultivating on ferrasols.

Key-Words: - Ash**,** *Bradyrhizobium japonicm*, CMC, ferrasols, organic matter, soybean

1 Introduction

It is generally accepted that soybean (*Glycine max* (L.) Merr.) originated in China. It has been introduced into other countries, the United States, Brazil, Argentina and Japan, which together with China constitute the major producers [1,2]. The protein content in soybean seed is approximately 40% and the oil content is 20%. This crop has the highest protein content and the highest gross output of vegetable oil among the cultivated crops in the world [3]. Also, soybean cultivation improves soil health because its ability to fix nitrogen fixation and its deep root [4].

Soybean [*Glycine max* (L.) Merr.] plays an important role in the global and agricultural nitrogen (N) cycles by facilitating biological fixation of atmospheric N into plant-available N when in symbiosis with rhizobia bacteria (*Bradyrhizobium* sp.). Soybean has been shown to fix an average of 175 kg N $ha^{-1}yr^{-1}$ in irrigated production, and 100 kg N $ha^{-1}yr^{-1}$ in dryland production [5]. Nitrogen fixation in soybean has been widely studied using different methodologies which reveal that soybean shows a strong demand for nitrogen (N) for optimal development and grain productivity [6,7,8]. Biological N fixation (BNF) accounts for 25 to 75% of the annual N uptake for the crop [9], with the remainder derived from mineral soil N.

Soybean is mainly nodulated by *Bradyrhizobium japonicum* and *B. elkanii* [10]. *Bradyrhizobium japonicum* strains are included on two types of carriers- peat and water. Peat (or humus) is used as a carrier in either a granular form, which is applied in-furrow, or in a powder form, which is applied to the seed at planting. Water-based products include liquid inoculants (seed applied or in-furrow) and frozen concentrates. New inoculants, introduced within the last 10 years, have increased potency due to sterile carriers and new packaging techniques [11]. Besides, there were varying reports on the interaction between variety and strain in soybean. Solomon *et al.* [12] and Thao [13] found a significant interaction between variety and strain on different parameters whereas Munyinda *et al.* [14] reported a no significant interaction.

Vietnam is the only country in the region experiencing double-digit growth in grain demand. Soybean milk requirement of Vietnamese is more and more with 613 million litres (2014)[3rd country in the world drink soy-milk] [15] and Vietnam imported over 1.2 million tonnes soybean seed. Soybean cultivation also introducted on red yellow latosol (ferralsols) in the begining of 1965-1970 with new cultivars from UDSA at EakMat experimental station at Ban Me Thuot [16]. An area of soybean cultivation of two provinces (DakNong and DakLak) of western highland of Vietnam reduced from 20.000 ha (2000) to 8.000 ha (2014) with 15.000 tonnes soybean production [15].

The aim of this study were (i) to evaluate the survaval of *Bradyrhizobium japonium* strain CJ02, (ii) to study the effects of carrier and protective material to *Bradyrhizobium japonium strain* CJ02 and (iii) to evaluate its effect on nodulation of soybean grown on ferralsols (experiment in pots).

2. Materials and Methods

Materials

Soil characteristics

The soil was ferralsols (or red latosol) in pH of 4.64, low in organic matter (3.575%), nitrogen total (0.135%) and available P_2O_5 (8.177 mg/kg) in the first experiment and pH=5.42, organic matter (3.822%), N total (0.137%) and available P_2O_5 (6.322 mg/kg) in the second experiment (Origin: Soil analysis Lab., Institute of Western Agriculture-Forestry Science [WASI]).

Rhizobial inoculant and carrier

Rhizobia strain: *Bradyrhizobium japonicum* strain CJ02 [17] was produced in YEM broth in 4 days, reached to $>10^9$ cell/ml.

Carrier consisted of sterile organic matter (50%), and ground black rice-hull ash (50%), carrier was ground with size 0.5 – 1 mm diameter, and the mixture was mixed with rhizobial liquid at moisture 25% (Carrier treatment). Rhizobial liquid mixed with 0.2% CMC [carboxyl methylcellulose] (treatment with CMC).

Rhizobial liquid, carrier and rhizobial inoculant with CMC were stored at 30°C in a box.

Experimental design

An experiment was arranged with completely randomized design with four replications, each pot (20x30 cm) was a treatment with 5 kg dry ferrasols (Figure 2), with 6 treatments as follows: T1 (control = no rhizobial inoculant), T2 (rhizobial inoculant with cover soybean seeds), T3 (rhizobial inoculant with CMC, with cover seeds), T4 (rhizobial inoculant with carrier, carriers mixed with seeds), T5 (T2 with ash covered soybean seeds when sowing), T6 (T3 with ash covered soybean seeds when sowing).

The experiment was carried out at 3 stages: 0, 15 and 45 days after rhizobial liquid, carrier, CMC… at the beginning of the storage at 30°C.

Yield component and Grain yield

Plant height and yield component were recorded at the time of maturity. Soybean seeds were harvested to calculate grain yield (gr/pot) after soybean seeds were dried at 70°C for constant weight. Soybean seed was also chosen to ground for determination of total nitrogen and oil contents.

Estimation of protein and oil

Total nitrogen content of soybean seed was determined by Micro-Kjeldahl method as recommended by AOAC, [18]. Nitrogen contents were multiplied by dry matter-based factor 5.71 to determined total protein content [19] and Oil content of soybean seeds was estimated by adopting Soxhlet Ether Extraction method [18].

Statistical Analysis

All the data pertaining to the present investigation were staticsally analyzed as per the method described by Gomez and Gomez [20]. The stastistically significant effect was tested at 1 per cent level of probability.

3. Results and Discussion

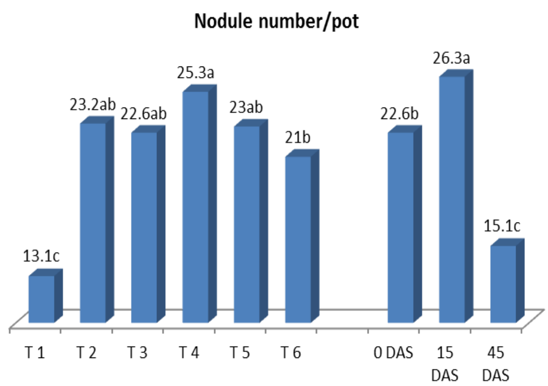
Survival and Nodulation of *B. japonicum* strain CJ02

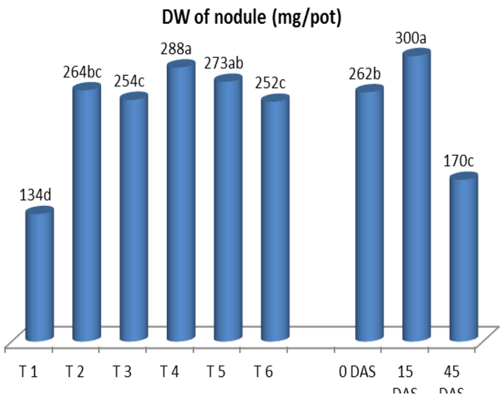
The good nodulation of soybean in control treatment (T1) showed that high population of native rhizobia in soil; however, the bradyrhizobial inoculant treatments had nodule number and DW of nodule/pot signifanictly higher than the control. This result demonstrated that *Bradyrhizobium japonicum* strain CJ02 nodulated the highest nodule numbers on soybean plants (cv. Cujut) and DW of nodule/plant [17] (Table 1) and bardyrhizobia also require many nutrients in ash and/or organic matter (in compost) to develop nodule and high DW of nodule in comparison with bradyrhizobial inoculant treatment without ash and/or organic matter (Figure 1), especially the good nodulation of soybean on 15 DAS stage compared to 0 and 45 DAS stage perhaps the role of ash and/or organic matter in the T4, T5 and T6 treatments supported the good survival of bradyrhizobia in the first stage of soybean seed (3 to 5 days after sowing) even though population of bradyrhizobia was high (Figure 2) (over 8 $\log_{10}$/ml). Wheareas CMC encouraged in good survival bradyrhizobial liquid during 45 days after storage at 30°C ($\log_{10}$/ml = 8.3) in comparison with bradyrhizobial liquid without CMC ($\log_{10}$/ml = 7.1) (Figure 2), carrier treatment (consisting of ash and organic matter) supported to survival of bradyrhizobia as well as prolonged the survival of bacteria during 45 days ($\log_{10}$/ml = 9.97). As a result, Bradyrhizobia not only survived from 1 to 3 after sowing but also flourished in the high population to promote good nodulation on young soybean roots during 3 to 7 days after sowing. Thus, good nodulation with high number of nodule/plant significantly supported grain yield of soybean, recorded to Table 3.

Table 1. Effecs of bradyrhizobial inoculant on nodulation of soybean (cv. Cujut) cultivated on ferrasols

Treatment	Nodule number/plant	Dry weigh of nodule/plant (mg)
0 day after storage (DAS)		
T1 (control)	13.00 e	131.25 d
T2 (Rhi liquid)	26.00 b	298.05 ab
T3 (Rhi+CMC)	29.50 a	330.25 a
T4 (Rhi in Carrier)	25.50 b	306.00 a
T5 (T2 with ash)	22.25 c	267.75 bc
T6 (T3 with ash)	19.50 d	241.00 c
Calculated F	**	**
15 DAS		
T1 (control)	17.25 d	177.50 d
T2 (Rhi liquid)	23.75 c	294.50 b
T3 (Rhi+CMC)	22.25 c	263.75 c
T4 (Rhi in Carrier)	36.75 a	390.75 a
T5 (T2 with ash)	33.75 b	419.75 a
T6 (T3 with ash)	24.00 c	278.75 c
Calculated F	**	**
45 DAS		
T1 (control)	9.00 d	94.75 d
T2 (Rhi liquid)	19.75 a	200.25 b
T3 (Rhi+CMC)	16.00 b	169.75 bc
T4 (Rhi in carrier)	13.50 c	169.25 bc
T5 (T2 with ash)	13.00 c	130.50 bc
T6 (T3 with ash)	19.50 a	254.75 a
Calculated F	**	**
C.V	**12.91%**	**9.27%**

Means within a column followed by the same letter/s are not significantly different at p<0.01





Note: T1: control, T2: Rhizobial liquid, T3: Rhizobial Liquid+CMC, T4: Rhizobial Liquid mixed in carrier, T5: T2 + Ash, T6: T3 + Ash

Figure 1. Effects of bradyrhizobia, CMC, Ash and organic matter (compost) on nodulation of soybean (cv. Cujut) cultivated on ferrasols (in pots experiment)

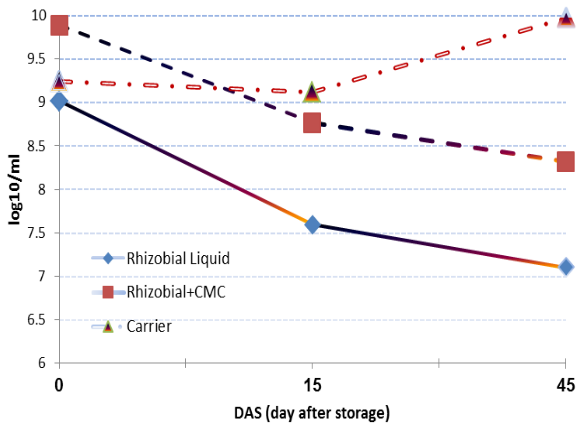


Figure 2. Effects of CMC, ash and organic matter on population of bradyrhizobia (log_{10}/ml) after 45 days stored at 30°C

Effects of *B. japonicum* on soybean cultivated on ferrasols

Application of bradyrhizobial inoculant affected on yield component of soybean cultivated on ferrasols positively especially plant height, total of pod/plant, number pod 2-seed/plant and 100-seed weight on 3 stages (0, 15 and 45 DAS) (Table 2a, 2b and 2c), this led grain yield of soybean were always higher than the control significantly (Figure 3). While the effectiveness of bradyrhizobial strain CJ02 decreased to the time (from 0 DAS to 45 DAS), CMC and ash promoted their survival and nodulation on soybean cultivated on ferralsols in three stages (0, 15 and 45 DAS) (Figure 3) and they also supported final grain yield of soybean (Figure 4).

Table 2a. Effects of Bradyrhizobial inoculant, CMC, Organic matter, Ash on Yield Component of soybean (cv. Cujut) cultivated on ferrasols at 0 DAS

Treat ment	Plant height (cm)	Pod total /plant	No of Pod bearing 1-seed/ plant	No of Pod bearing 2-seeds/ plant	No of Pod bearing 3-seeds/ plant	100-seed weight (gr)
Control	91.7 b	13.75 c	2.25 c	8.0 d	1.25 c	13.89 c
Rhizo	118.7 a	29.50 ab	7.00 a	16.0 ab	1.75 bc	14.90 ab
Rhizo +CMC	118.5 a	19.00 bc	3.25 b	11.0 c	4.00 a	15.17 a
Carrier	111.7 a	23.50 b	6.50 ab	13.5 b	2.75 b	14.98 a
Rhizo +Ash	122.0 a	20.25 bc	4.75 ab	12.2 bc	2.25 bc	14.54 b
Rhizo+ CMC +Ash	112.7 a	32.0 a	7.75 a	18.7 a	2.50 b	15.21 a
Cal. F	**	**	**	**	**	**
C.V (%)	**5.59**	**18.49**	**37.56**	**15.56**	**36.49**	**1.35**

Means within a column followed by the same letter/s are not significantly different at p<0.01

Table 2b. Effects of Bradyrhizobial inoculant, CMC, Organic matter, Ash on Yield Component of soybean (cv. Cujut) cultivated on ferrasols at 15 DAS

Treat ment	Plant height (cm)	Pod total /plant	No of Pod bearing 1-seed/ plant	No of Pod bearing 2-seeds /plant	No of Pod bearing 3-seeds /plant	100-seed weight (gr)
Control	88.2 b	7.5 d	1.50 b	4.5 d	0.75	14.04 c
Rhizo	99.7 b	12.2 c	0.75 b	10.5 b	0.75	15.78 a
Rhizo +CMC	90.0 b	11.0 c	2.25 b	7.7 c	0.75	15.63 a
Carrier	116.2 a	20.0 a	5.50 a	12.7 ab	1.75	15.88 a
Rhizo +Ash	98.7 b	16.2 b	2.75 b	10.5 b	2.50	15.66 a
Rhizo+ CMC+ Ash	91.2 b	18.5 ab	2.25 b	14.0 a	2.00	15.10 b
Cal. F	**	**	**	**	**n.s**	**
C.V (%)	**6.53**	**9.86**	**48.99**	**12.58**	**81.51**	**1.00**

Means within a column followed by the same letter/s are not significantly different at p<0.01
n.s = not significant

Table 2c. Effects of Bradyrhizobial inoculant, CMC, Organic matter, Ash on Yield Component of soybean (cv. Cujut) cultivated on ferrasols at 45 DAS

Treat ment	Plant height (cm)	od total /plant	No of Pod bearing 1-seed /plant	No of Pod bearing 2-seeds /plant	No of Pod bearing 3-seeds /plant	100-seed weight (gr)
Control	86.7 b	8.2 c	3.00	3.00 c	0.25	13.13 d
Rhizo	118.2 a	11.0 b	3.00	5.25 b	1.00	14.27 c
Rhizo +CMC	116.0 a	11.7 b	3.50	5.75 b	0.00	14.64 b
Carrier	118.5 a	15.0 a	4.25	7.75 a	1.00	14.69 b
Rhizo +Ash	117.2 a	14.0 a	3.50	6.00 b	1.00	14.77 b
Rhizo +CMC +Ash	98.5 b	13.5 ab	4.50	5.75 b	1.00	15.40 a
Cal. F	**	**	n.s	**	n.s	**
C.V (%)	**5.78**	**9.13**	**37.77**	**15.22**	**118.82**	**0.88**

Means within a column followed by the same letter/s are not significantly different at p<0.01
n.s = not significant

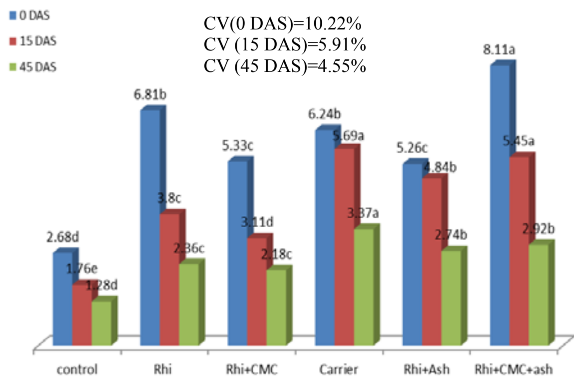


Figure 3. Effects of bradyrhizobium, CMC, organic matter and ash to grain yield of soybean (cv. Cujut) cultivated on ferralsols in three stages [0, 15 and 45 DAS] (in pots experiment)

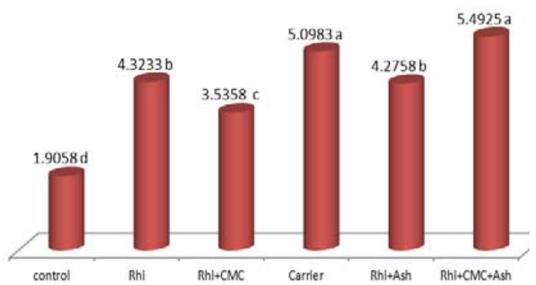
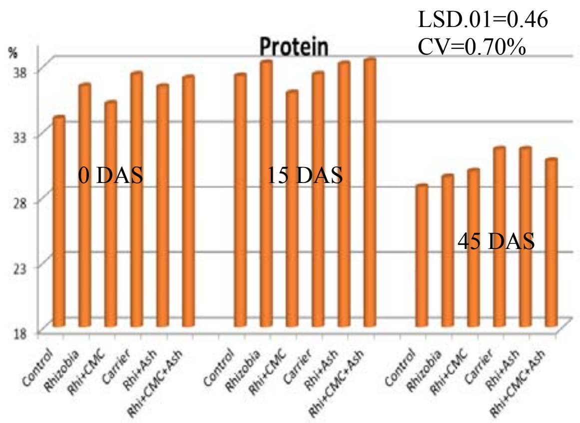


Figure 4. Effects of bradyrhizobium, CMC, organic matter and ash to grain yield of soybean (cv. Cujut) cultivated on ferralsols (in pots experiment)

Besides, DW of nodule correlated grain yield of soybean in 3 stages (0, 15 and 45 DAS) (Table 3) very significantly especially in 0 DAS. Regression equation having the highest value in comparison with 15 and 45 DAS showed that the survival of bradyrhizobia affected to grain yield of soybean. Furthermore, protein concentration (%) in soybean seed in 45 DAS was the lowest (Figure 5a) but lipid concentration in soybean seed in the treatments supplementing CMC, ash and/or organic matter were higher than treament of bradyrhizobial inoculation alone (Figure 5b). This demonstrated that the important role of CMC, ash and organic matter in supporting and providing the nutrients for the survival of bradyrhizobia and the growth of soybean.

Table 3. The relationship between DW of nodule with grain yield of soybean cultivated on ferralsols (in pots experiment)

DW of nodule (mg/pot)	Grain yield of soybean (g/pot)
0 DAS	$y = 20.834x + 142.93$ $r = 0.541$**
15 DAS	$y = 0.0111x + 0.7697$ $r = 0.683$**
45 DAS	$y = 0.0067x + 1.3415$ $r = 0.526$**



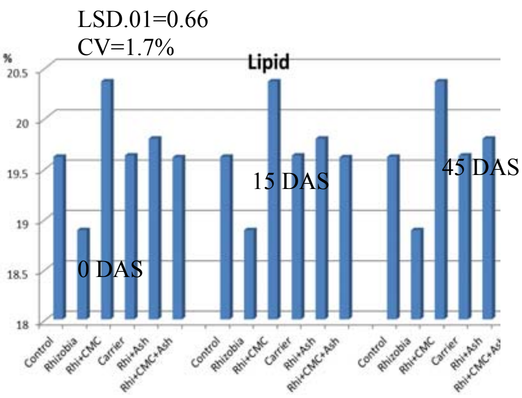


Figure 5. (A) Effects of bradyrhizobium, CMC, organic matter and ash to protein concentration (%) and (B) lipid concentration in soybean seed cultivated on ferrasols (in pots experiments)

Hume and Blair [21] found that nodule number and mass, as well as seed yield, increased curvilinearly upward with increasing $\log_{10}$ most probable numbers (MPNs) of *B. japonicum*; Papakoska [22] showed linear relationship between the rate of applied rhizobia and the number of the nodules per plant or the dry weight per nodule; Maurice *et al.* [23] with commercial liquid inoculants, demonstrated that *B. japonicum* cells from old inoculants needed more time to divide and produce visible colonies, indicating that they would probably be unable to grow in soil. Survival of *B. japonicum* cells strain CJ02 was declined to by the time [from log10/ml: 9.00 to 7.10 at 45 days after storage at 30^{o}C] and their survival were improved by supporting of CMC able to keep moisture. In addition to this, carrier with combination of organic matter and ash not only made a good microenvironment for their survival but also provided nutrient for their growth. As aresult, their population increased from $\log_{10}$/ml: 9.20 (0 DAS) to $\log_{10}$/ml: 9.89 (45 DAS) and this result demonstrated again the effect of ash on their survival of rhizobia [24].

Competition between the rhizobia in the inoculant and the microbes already in the soil, the native microflora, affects the success of many inoculants; soil contains billions of different bacteria and other microorganisms, and the process of seed inoculation adds billions more; this causes immediate competition between the microbes, as they are all fighting for the same resources, if the rhizobia in the inoculant can out compete the native population, inoculation will be successful, nodules will form, and they will begin nitrogen fixation [25]. To surface-sterilize soybean seeds before sowing, Oerhre *et al.* [26] used penicilin G with 500 mg/l because penicillin G did not reduce seed germination and root tip growth or affect to growth seedling development. Penicillin G treament of soybean seed seedlings can be used to reduce root colonizing microbes but it did affect to *B. japonicum* to the soybean root, the result showed that the survive of *B. japonicum* 2143 and their population at soybean root increased from 2005 to 325%. *Bradyrhizobium japonicum* strain CJ02 was isolated from nodules of soybean cultivating at ferrasols [17], it survives good in ferrasols in long time (more 30 years) and Dak-Nong ferrasols has two seasons in a year: dry season (from November to April) and wet season (May to October) and bradyrhizobia should survive during dry season without soybean plants. Interestingly, the transcriptional analysis of the genome of *B. japonicum* subjected to desiccation stress indicated that genes critical for pilus assembly, e.g., pilA, pilA2, and ctpA, are upregulated [27]. Pili, especially type IV pili, are often important for biofilm formation [28];[29]. It is extremely likely that desiccation-stressed *B. japonicum* cells show some of the same patterns of gene expression, as do cells in biofilms. Our results showed that CMC (carboxyl methyl-cellulose), ash and organic matter supported and provided nutrients for good nodulation of soybean and for growth of soybean plants.

4 Conclusion

Carboxyl methyl-cellulose (CMC), ash (by-product from cooking) and organic matter (compost) affected on survival of *Bradyrhizobium japonicum* strain CJ02, good nodulation (number of nodule and dry weight of nodule) significantly; this result promote to rise plant height, shoot dry weight, pod dry weight, yield, and quality of soybean seed compare to the control and *Bradyrhizobium* inoculation. growth and yield of soybean (cv. Cujut) cultivated on ferrasols. However, *Bradyrhizobium* inoculation significantly increased plant height, shoot dry weight and pod dry weight compared with the control. Application of *Bradyrhizobium* [with CMC] inoculation plus ash and organic matter could be a useful practice in sustaining the growth and yield of soybean cultivating on ferrasols.

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