

Technical Report

**Rapid Measurement of Hydrogen Concentration and Its Use in the Determination of Nitrogenase Activity of Legume Plants**

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**Abstract**

A method and apparatus for rapidly measuring H<sub>2</sub> concentration in a gas stream and the use of this instrument to measure nitrogenase activity of a nitrogen fixing plant growing in a non-porous container is described. The method does not require that the plants be sealed in their growth pots for gas exchange measurements, but allows these measurements to be made by sampling H<sub>2</sub> (a by-product of N<sub>2</sub> fixation) directly from the medium in which the plants are grown. Gas containing hydrogen is sampled at the surface of the medium at a rate approximately equal to the rate of hydrogen diffusion from the medium, and the sample is passed to a hydrogen sensor where it is measured and nitrogenase activity determined therefrom. This allows for very rapid measurements of nitrogenase activity involving little or no manipulation of the plant material being studied. As a result, large populations of plants may be screened quickly for variations in nitrogenase activity. This method would be especially useful for assessing variations in nitrogenase activity caused by application of different environmental treatments to a specific legume-*Rhizobium* symbiosis.

Keywords: H<sub>2</sub>, nitrogenase activity, nitrogen fixation rate

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## 1. Introduction

The yield of many crop species is limited by the amount of nitrogen available in the soil. To alleviate this limitation, farmers supplement the soil with nitrogenous fertilizers that are expensive and hazardous to the environment. Some important crop species such as soybean, pea, alfalfa, clover and bean do not rely entirely on soil nitrogen but are able to meet their requirements by reducing atmospheric  $N_2$  to ammonia through a process called  $N_2$  fixation carried out by bacteria which form a symbiotic association with the roots of the host plant. Biological  $N_2$  fixation is likely to become an essential component of sustainable agricultural systems, and a great deal of research is currently in progress to investigate the genetics of nodule formation and to determine the factors which regulate  $N_2$  fixation in leguminous crops. Consequently, a simple, accurate method is required for providing a non-invasive measurement of the rate of  $N_2$  fixation.

The reduction of atmospheric  $N_2$  to ammonia is catalyzed by the enzyme nitrogenase and the activity of this enzyme therefore determines the  $N_2$  fixation rate. Several methods have been devised to measure the  $N_2$  fixation rate and/or nitrogenase activity of legumes. These methods include measurement of whole plant nitrogen increment,  $^{15}N$  natural abundance assay,  $^{15}N$  enrichment assay (Burris, 1942; 1991), acetylene reduction assay (Hardy et al., 1968; Vessey, 1994) and  $H_2$  evolution assay (Simpson and Burris, 1984; Hunt and Layzell, 1993).

The measurement of  $H_2$  evolution using a  $H_2$  analyzer in the open system assay (Layzell et al., 1984) has several advantages over other methods for measuring nitrogenase activity and  $N_2$  fixation rate: The  $H_2$  analyzer is extremely sensitive and it is the only instrument which allows continuous, real-time measurement of nitrogenase activity. The  $H_2$  analyzer is the only instrument that allows measurement of apparent nitrogenase activity (ANA), total nitrogenase activity (TNA), and  $N_2$  fixation rate on the same plant material. Measurements can be performed on the same plant material either continuously or intermittently over virtually any experimental period. The method is not labor intensive and the  $H_2$  analyzer is much cheaper than the mass spectrometer required for  $^{15}N$  measurements or the gas chromatograph required for acetylene reduction assay.

Despite these advantages, relatively few researchers use the  $H_2$  evolution assay to measure nitrogenase activity. One of the main factors that discourages people from using the  $H_2$  evolution assay is that current methods for the assay require that plants be sealed in their growth pots to allow  $H_2$  to accumulate before analysis (closed system assays), or to allow nodulated root systems to be flushed with specific gas mixtures (open system assays). The procedure is time-consuming, and at least 5 minutes is required before stable measurements can be

obtained. Also, the pot must be unsealed after the assay is completed if further growth and development of the plant is to be studied. This increases the time required to conduct the measurements and limits the number of plants that can be assayed within a working day. A further limitation to the use of H<sub>2</sub> assays is that it requires the use of sophisticated instrumentation, and the supply of specific gas mixtures to the material being studied. As a result the assays can only be conducted in laboratories within research institutions, or in mobile laboratories constructed in the field.

These limitations of the H<sub>2</sub> assays make it unsuitable for the large scale screening of plants that is needed to identify legumes with specific N<sub>2</sub>-fixing characteristics. At present, much of the research in the field of N<sub>2</sub> fixation involves genetic manipulation of legumes, and assessment of the effects of such manipulation on nitrogenase activity. This approach requires the growth of large populations of genetically altered plants (usually in a greenhouse) and assessment of their nitrogenase activities under various environmental conditions. To make appropriate statistical analyses of the data, the plants must be screened at the same stage of development at the same time of day. It should be apparent, therefore, that there is need for a rapid method of nitrogenase activity analysis that can be performed in a greenhouse setting.

This study reports on the design and use of a portable instrument by which nitrogenase activity can be measured rapidly in H<sub>2</sub>-producing legumes by sampling H<sub>2</sub> from the medium in which the plants are grown without the necessity for sealing the plants within their growth pots.

## 2. Methods

### *The H<sub>2</sub> analyzer*

A portable H<sub>2</sub> analyzer was designed to measure H<sub>2</sub> concentration in field or greenhouse conditions (Fig. 1). A pump (Brey Model GO1 ASF Industries, Norcross, GA, USA) pulls gas into the instrument through an external column filled with a magnesium perchlorate drying agent. The dry gas passes through a filter to remove particulate matter, and then through a H<sub>2</sub> sensor (Model 822, Figaro Engineering Inc., Osaka, Japan) enclosed within a temperature-insulated block. The sensitivity of the H<sub>2</sub> sensor alters with temperature, and since the instrument was designed to use in the field or greenhouse where environmental conditions vary, it is essential to maintain a constant sensor temperature. This is achieved by heating the sensor with a proportional integral derivative (PID) controlled heating device. A sensor monitors the temperature of the insulated block and the signal from this sensor feeds into the PID controller to maintain a constant temperature. The flow rate of gas through the instrument is

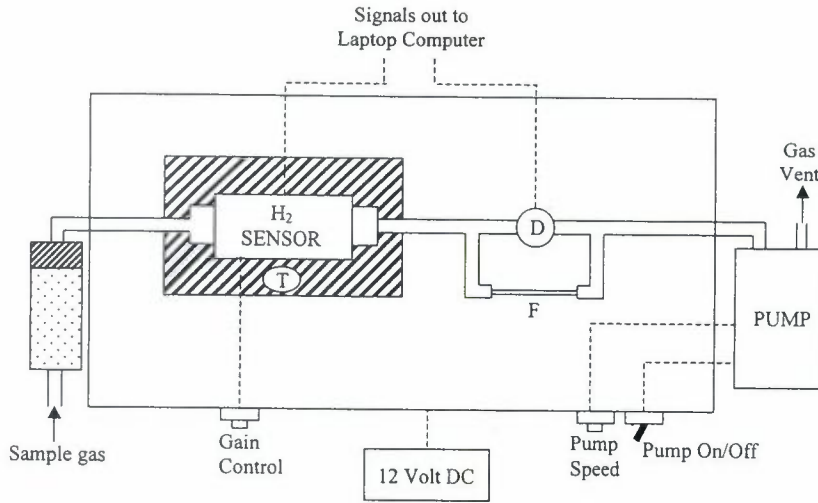


Figure 1. The schematic diagram of the H<sub>2</sub> analyzer and the embodiment for H<sub>2</sub> production rate measurement. D: differential pressure sensor; F: flow restrictor; T: temperature sensor in PID-controlled block.

monitored by measuring the pressure drop across a flow restrictor in the gas line using a differential pressure sensor (Model MPX10DP, Motorola Corporation, USA). Flow rate can be altered by varying the pump speed using a potentiometer. Outputs from the H<sub>2</sub> and differential pressure sensors are monitored, preferentially, by a portable computer, although a 2 channel analog recording device may be used. Power for the pump, heater and the sensors is supplied by an external 12 V battery. The gas-sampling head of the instrument consists of a 1/8 inch (i.d.) LUER® fitting attached to flexible 1/8 inch (i.d.) Tygon tubing.

#### *Plant material*

Lupin and soybean plants were grown in coarse silica sand (Grade 16) in growth chamber with 16 hr day at 25°C and 8 hr night at 17°C. Rhizobia strains without uptake hydrogenase (HUP-) were used to inoculate the plants. Plants of different age were used to measure the hydrogen concentration on the surface of sand and nitrogenase activity of nodules.

#### *Collection of sample gas*

The method of measuring nitrogenase activity using the portable H<sub>2</sub>

analyzer is very different from that using open-flow gas exchange analysis. The portable H<sub>2</sub> analyzer takes a sample of gas directly from the surface of the planting medium in which the legume is grown, without the need for sealing the pot. This is achieved by attaching the sampling head to a novel sampling-cap that fits on the surface of the planting medium. The sampling cap is a plastic disc with a slot for accommodating the stem of the plant, and a lip 0.5 cm deep around its perimeter that is pushed into the planting medium before sampling (Fig. 1). The cap allows H<sub>2</sub> to be sampled from a greater area of the planting medium than if the sampling head alone were used. This is important because H<sub>2</sub> evolution from the planting medium is not uniform but is often greatest closer to the stem, since the crown region of the root, at the root-stem junction, has the greatest concentration of H<sub>2</sub> evolving nodules. Use of the sampling cap therefore reduces the variation that would occur in measurements of samples collected over a smaller surface area of the planting medium.

#### *Calibration and quantitative measurements of nitrogenase activity*

To make quantitative measurements of nitrogenase activity it is essential that the H<sub>2</sub> sensor is calibrated. This is achieved by pumping gases containing known concentrations of H<sub>2</sub> through the analyzer while monitoring H<sub>2</sub> sensor output. Hydrogen was mixed with air by using Wosthoff pumps (Calibrated Instruments Inc., Hawthorne, NY, USA) or by an electrolytic H<sub>2</sub> generating device designed specifically for H<sub>2</sub> analyzer calibration (NAAS Calibrator, Morgan Scientific, Haverhill, MA, USA).

The next stage of the calibration procedure occurs as plants are being screened for high and low nitrogenase activities. The sampling cap is placed around the stem of a plant, and the H<sub>2</sub> concentration in the gas at the surface of the planting medium is measured using the H<sub>2</sub> analyzer. The tubing from the sampling cap is then disconnected from the analyzer, and the sampling cap is removed from the surface of the planting medium. The input of the H<sub>2</sub> analyzer is connected to a pump that sucks air from the base of the pot via a metal tube inserted through a drainage hole (Fig. 2). Other drainage holes in the pot are plugged. Outside air is drawn from the top to the bottom of the pot and all the H<sub>2</sub> produced by the plant is sampled by the pump. The flow rate at which gas is drawn through the pot is measured by a flow meter and maintained at 2L/min. An in-line water trap prevents nutrient solution from the pot entering the pump. The pump incorporated in the H<sub>2</sub> analyzer sub-samples the gas from the external pump, and the H<sub>2</sub> concentration of the gas stream is measured. The rate of H<sub>2</sub> production from the plant can then be calculated using the following equation:

$$\mu\text{mole H}_2/\text{hr} = (\text{pH}_2 * 0.164 * \text{FR} * P) / (R * T) \quad (1)$$

where  $p_{H_2}$  is in units of ppm,  $FR$  = gas flow rate through the pot in mL/min,  $R$  = gas constant of 22.4136 L/mol at 273K and 100 kPa pressure,  $T$  = temperature in K, and  $P$  = ambient pressure in kPa. This procedure is repeated for several plants which have different  $H_2$  concentrations at the surface of the planting medium, so that a relationship is established between the  $H_2$  concentration measured by the sampling cap and the rate of  $H_2$  evolution measured by sampling the gas from the base of the pot.

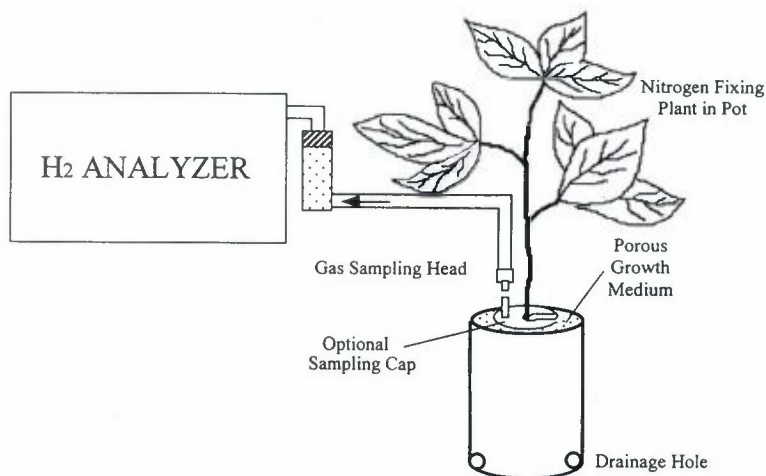


Figure 2. A diagram showing the use of the portable  $H_2$  analyzer to measure  $H_2$  evolution rate (nitrogenase activity) from a  $H_2$ -producing legume plant.

### 3. Results and Discussion

#### *Qualitative measurements of nitrogenase activity*

For qualitative measurements of nitrogenase activity, in which activity is to be compared among individual plants in a population, calibration of the  $H_2$  sensor is not essential. To make these measurements of nitrogenase activity the analyzer pump is set at a constant flow rate (typically 30 ml/min), and the sampling head is attached to the cap. Normal air is sampled to obtain a zero reading from the  $H_2$  sensor, and then the cap is placed around the stem of the plant as shown in Fig. 1. Gas from the surface of the growth medium is sampled until a stable output from the  $H_2$  sensor is observed (usually within 45 seconds). The procedure is then repeated on the next plant. Since the voltage output from

the H<sub>2</sub> sensor is proportional to the amount of H<sub>2</sub> evolved from the plant during N<sub>2</sub> fixation, plants can be screened for high or low nitrogenase activities on the basis of relative voltage measurements alone. An example of a set of relative nitrogenase measurements made on a population of lupin plants is shown in Fig. 3.

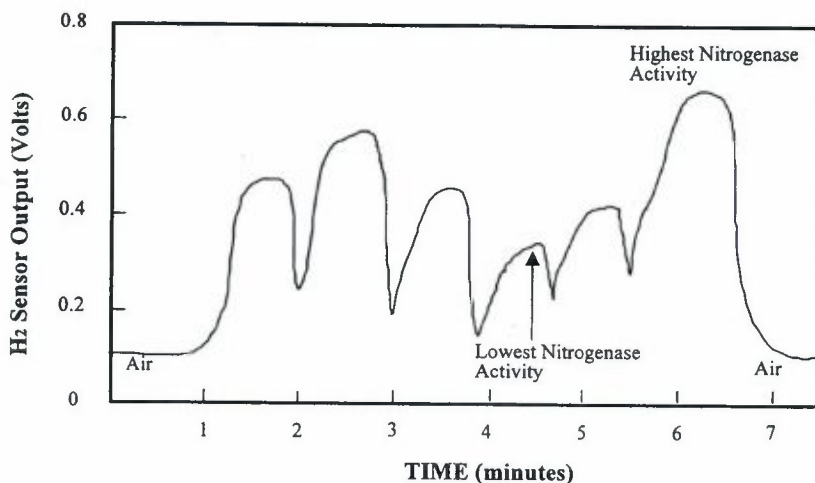


Figure 3. Typical data from the portable H<sub>2</sub> analyzer when it is used to measure H<sub>2</sub> production from 6 lupin plants in sequence. Highest and lowest relative nitrogenase activities detected by the analyzer are shown.

It is important when using this method to ensure that plants are grown in pots with uniform dimensions and in the same planting medium, since pot shape and size, and the diffusion characteristics of gases through different media, can affect the efficiency of H<sub>2</sub> capture by the sampling cap. The planting medium must be free draining so that water does not restrict H<sub>2</sub> diffusion, and a medium with the porosity of coarse silica sand (Grade 16) is recommended. Even in such a medium, measurements should not be taken within 60 minutes of watering the pot. Soil should not be used as a planting medium for two reasons. First, it retains water, and second it contains microorganisms that have the capacity to oxidize H<sub>2</sub> (La Favre and Focht, 1983). The presence of H<sub>2</sub>-oxidizing microorganisms in the planting medium will lead to underestimates of nitrogenase activity, and it is recommended that all planting media should be sterilized before cultivation of the legumes.

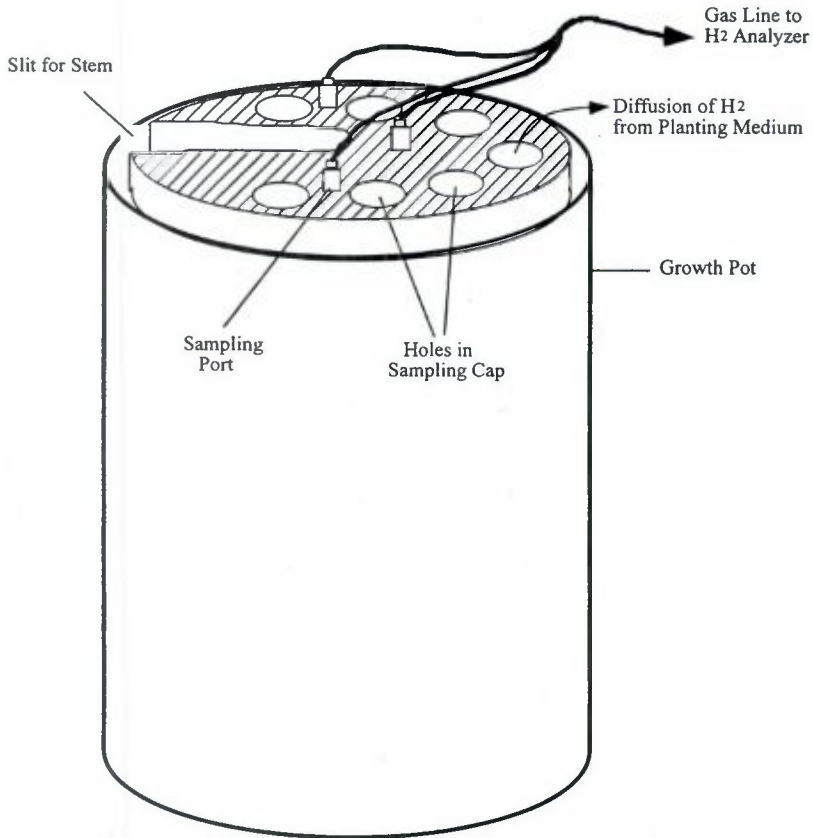


Figure 4. A diagram showing an alternative embodiment of the soil gas sampling cap.

The  $H_2$  on the surface of planting medium was the result of diffusion. Under steady state conditions the diffusion flux across the surface of the pot equal the  $H_2$  production rate of the nodules. This rate was determined by measuring the flow rate in a open system and  $H_2$  concentration in the effluent gas stream. The comparison of the  $H_2$  concentration on the planting medium surface by diffusion and the  $H_2$  production rate of the plant showed that when plants are grown in gas-impermeable pots open to the atmosphere,  $H_2$  diffusion flux out of the pot due to diffusion is equivalent to 100–150 ml/min at 20°C. Therefore, gas cannot be sampled from the surface of the medium at a greater flow rate without depleting  $H_2$  at the surface and altering steady state conditions. However, covering the surface of the sampling medium with the sampling cap may also disturb the diffusion characteristics of  $H_2$  and lead to erroneous results. Balance



must be struck between the need to sample H<sub>2</sub> from a large surface area of the planting medium and the need to prevent changes in H<sub>2</sub> diffusion. Our experiences showed that the sampling cap should cover not more than 60% of the surface of the planting medium, allowing H<sub>2</sub> to diffuse freely from the remaining surface during the sampling period. In one embodiment of the cap, as shown in Fig. 1, the cap is a solid disc of plastic with a slit that accommodates the stem of the plant, and with a single gas outlet port connected to the H<sub>2</sub> analyzer. In an alternative embodiment (Fig. 4), the disc covers almost the entire surface of the planting medium, but contains holes accounting for 40% of the area of the medium, through which H<sub>2</sub> diffuses to the atmosphere. In this embodiment, there may be several gas outlet ports through which H<sub>2</sub> is sampled, these combining into a single gas stream that enters the analyzer. Using either embodiment of the sampling cap, at a sampling flow rate of 30 ml/min, the steady state H<sub>2</sub> concentration within the sample can be measured within 45 seconds, during which time H<sub>2</sub> diffusion from the soil remains stable.

It should be noted that the sampling cap is not designed to capture all the H<sub>2</sub> evolved from the nodulated root. Only a portion of the total H<sub>2</sub> evolved from the surface of the planting medium and the cap may differ in dimension depending on the size of the pot in which it is used. For a given population of plants grown in pots with the same dimensions, use of a standard sampling cap increases the accuracy of relative measurements of nitrogenase activity, or, if the H<sub>2</sub> analyzer is calibrated as described below, increases accuracy of quantitative measurements of nitrogenase activity.

#### *Quantitative measurements of nitrogenase activity*

Hydrogen concentration on the surface of planting medium and the total hydrogen production rate of the same plant were measured by sniffing through the sampling cap and suck air from the bottom of the pot. Several soybean plants of different age were used to find the relationship between the hydrogen concentration on surface of the planting medium and the nitrogenase activity. This relationship is linear, as shown in Fig. 5. Once the relationship is established it can be used to determine H<sub>2</sub> production rate by the nodulated roots from a very quick and simple measurement of surface H<sub>2</sub> concentration using the sampling cap. The relationship needs not to be established prior to screening a large population of plants, but individual plants with various surface H<sub>2</sub> concentrations can be selected for rate measurements during the screening process.

Quantitative measurements of H<sub>2</sub> evolution rate, as described above can only be made if the plants being screened are all grown in pots of uniform dimension containing the same planting medium. The cautions regarding the planting

medium described above for the qualitative measurements of nitrogenase activity also apply to quantitative measurements.

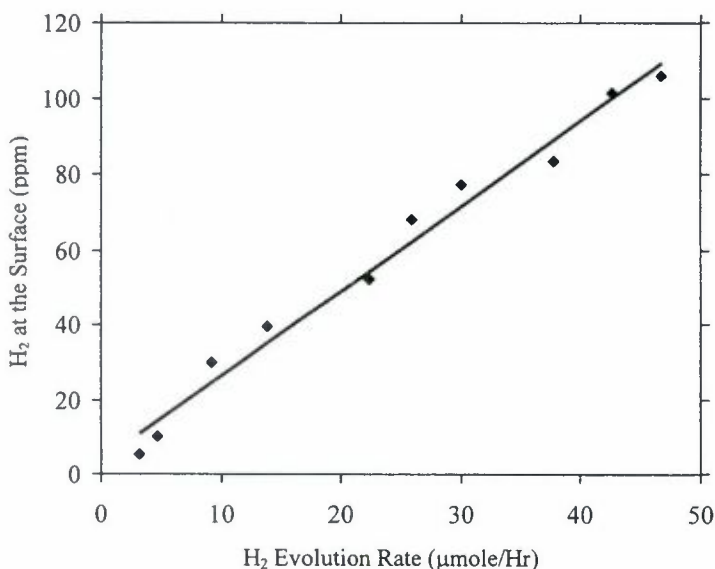


Figure 5. The relationship between H<sub>2</sub> concentration measured at the surface of the planting medium in pots containing H<sub>2</sub>-producing soybeans, and the rate of H<sub>2</sub> production (nitrogenase activity) from the nodulated roots of the plants.

#### 4. Conclusion

For rapid measurements of physiological processes by gas exchange, standard methods of open flow gas analysis are not appropriate. In particular, the time-consuming task of sealing and unsealing pots containing the material under study must be avoided. For example, when screening large populations of plants for variations in nitrogenase activity the time constraints involved in standard methods of H<sub>2</sub> analysis would allow only a small proportion of the population to be screened in a single working day. In these investigations it is often more important to rapidly identify plants within a population that have nitrogenase activities significantly higher or lower than the rest of the population. For this purpose, only apparent nitrogenase activity (ANA) needs to be determined, which requires measurement of H<sub>2</sub> evolution in the air alone. Therefore, the gas mixing components, and much of the calibration procedures,

of the standard method are unnecessary. The new instrument and method circumvents this limitation and allows measurements of ANA to be conducted in a fraction of the time required for standard open flow ANA measurements. A new calibration procedure has also been developed to make the ANA measurements quantitative. The portability of the instrument allows for screening of large plant populations outside of the laboratory.

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