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Research Article

PLANT GROWTH-PROMOTING BACTERIA IN COMBINATION WITH HUMIC ACIDS IMPROVE GROWTH OF WHITE CLOVER (*TRIFOLIUM REPENS L.*) CULTIVATED IN POOR SOILS

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Summary: The main objective of this study was to investigate the possibilities for improving the plant growth of *Trifolium repens L.* (white clover) cultivated in poor of nutrients soil. The effects of mixed cultures of seven strains of rhizospheric microflora belonging to the genera *Bacillus* and *Pseudomonas* and/or humic acids on the basic biometric parameters of the test plants were evaluated. Soils with low humus and nitrogen contents were used as substrates. Treatment with humates showed a strong positive effect on plant growth. The observed increase of fresh and dry weight of the aboveground biomass was by 77% and 120%, respectively. The best results were obtained after application of a combination of rhizospheric microflora and humic acids resulting in an increase of plant dry biomass by 160% compared with the control.

Keywords: Humic acids; Plant Growth-Promoting Rhizobacteria; Reclamation; *Trifolium repens* L.

Abbreviations: HA – Humic Acids, HS – Humic Substances, PGPR – Plant Growth-Promoting Rhizobacteria.

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INTRODUCTION

Nowadays, the environmental contamination caused by the mining activities needs a complex approach. A priority is given to the full recultivation of the affected areas, as the methods of reclamation are directly related to the nature of the violations. A final aim is the restoration of the ecosystem in the last biological phase of the recultivation - a complex of meliorative and bioremediative measures, focused at restoring and improving both the structure and fertility of the soil. This includes the management of all kinds of physical, chemical and biological soil disorders, such as soil pH, fertility, microbial communities and nutrient cycles in the soil. When using non-humus reclamation technology or organic-poor soils, the three basic biogenic elements, namely nitrogen, phosphorus

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and potassium, are usually not sufficiently available (Coppin and Bradshaw, 1982; Sheoran et al., 2008). Besides, the optimal vegetation growth is observed at neutral pH. All relatively newly formed mining soils, as well as old eroded and degraded soils, require an abundant fertilization and an increase of the soil pH in order to create and maintain a sustainable plant community in the terrains that are impacted anthropogenically because of mining and/or processing industries.

One of the alternatives of conventional techniques and bioremediation is focused on plant growth-promoting rhizobacteria (PGPR) - microorganisms that colonize the roots of phytoremediatior plants, i.e. the rhizosphere - the zone of soil, surrounding the roots. There are many reports on plant growth promotion and yield enhancement by PGPR (Lugtenberg et al., 2009; Bratkova et al., 2012 a, b; Karlicic et al., 2016).

Bacterial fertilizers show significant advantages over synthetic ones due to the ability of rhizospheric bacteria to synthesize a wide range of physiologically active substances such as vitamins, auxins, gibberellins, cytokinins, etc. They can influence the activity of useful microorganisms thus enhancing photosynthesis and facilitating the enzymatic processes in plants. In addition, they act as antagonists against pathogens and phytopathogens (Licheva et al., 2011). The application of bacterial fertilizers with highly efficient rhizospheric microflora supports the mineral nutrition of plants thus promoting the process of biological reclamation (Kaisheva et al., 2015).

The new trends in environmentally friendly and sustainable management of disturbed terrains highlight the synergistic effects associated with inoculum, consisting of several mixed strains. Symbiotic bacteria (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and several non-symbiotic bacteria (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillum, Azomonas*) have been used in the production of bio-inoculants (Ahemad and Kibret, 2014). These bacteria have a positive effect on the plants subjected to various stress conditions such as salinization (Qurashi and Sabri, 2012), heavy metals contamination (Hao et al., 2012), and xenobiotic (Ahemad and Khan, 2012). According to Georgieva et al. (2018), some *Pseudomonas* strains belonging to different species are able to solubilize nutrients, to produce different metabolites and enzymes, and to act as antimicrobial agents.

The humic substances (HSs) as a major components of soil organic matter are among the most complex and biologically active compounds that stimulate both plants and microbial activities through a number of mechanisms (Canellas and Olivares, 2014; Ekin, 2019). Having unique physiochemical and biochemical properties, HS promote the physiological and biochemical processes in plants by triggering multiple interconnected signaling pathways. Crops treated with HS are highly tolerant to environmental stresses (Shah et al., 2018). The HS also might have both direct and indirect effects on plant growth and mineral assimilation (Chen and Aviad, 1990). Indirect effects involve improvement of soil properties such as aggregation, aeration, permeability, water holding capacity, ions transport and availability through

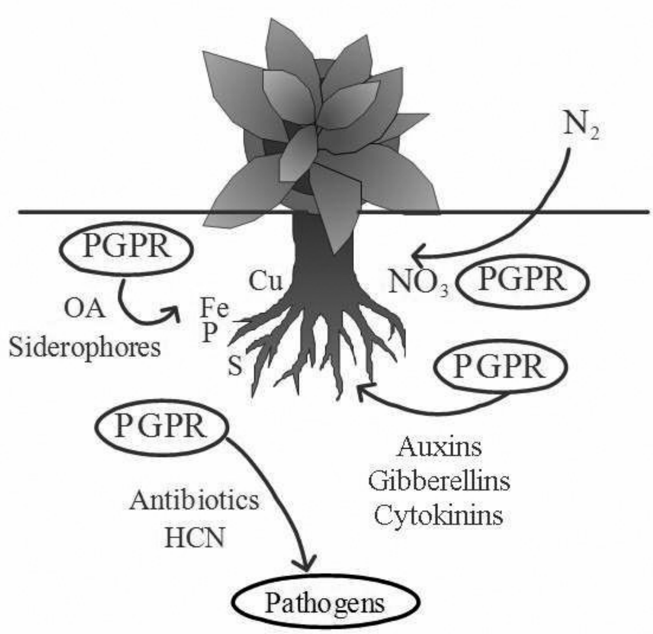

Figure 1. PGPR mechanisms to enhance interactions in the rhizosphere for higher yield and nutrient content (Ramakrishna et al., 2019).

pH buffering. HS have an abundance of carboxyl groups and weakly acidic phenolic groups, which contribute to their complexation and ion-exchange properties. They exhibit both hydrophobic and hydrophilic characteristics and can bind to soil mineral surfaces (Mikkelsen, 2005). Humic mateials are able to complex various cations and serve as a sink for polyvalent cations in the soil.

Humic acids (HA), extracted from brown and lignite coals, are widely used in the world practice for restoration of terrains disturbed by the extraction of minerals. Humic acids have the ability to make complexes with heavy metals and radionuclides. Under certain conditions they form with the latter insoluble complexes, thus preventing their migration and the translocation of pollutants. Literally, HA have high adsorption capacity and great specific surface area. The HA contribute to the rapid restoration of the fertility of treated substrates (Petrova et al., 2016).

Due to a reduction of chemical inputs, improvement of diversity, fertilization management, integrated nutrient management, the use of beneficial soil microorganisms supplemented with HS is one of the popular techniques that aims sustainable agriculture (Shah and Wu, 2019). Ekin (2019) has reported a significant improvement of plant production (of potato as a test plant) through a co-inoculation with *Bacillus* strains and humic acid.

The main objective of this study was to investigate the possibilities for improving the plant growth and development of white clover (*Trifolium repens* L.) cultivated in soil with low humus and nitrogen content. The effects of the applied rhizospheric microflora and/or humic acids on the basic biometric parameters of the test plants were evaluated.

MATERIALS AND METHODS

Experimental design

Laboratory experiments with white clover *(Trifolium repens L.)* cultivated in soil poor of nutrients were performed. Containers (14 cm high) having a volume of 5 dm^3 were filled with soil. In each pot 0.2 g of white clover seeds were sown.

The experimental scheme included 4 types of treatment: 1 – Control; 2 – Treatment with rhizospheric bacteria (PGPR); 3 – Treatment with humic acids (HA); 4 – Combined treatment (rhizospheric microflora + humates, PGPR + HA). Treatments were repeated three times, in three consecutive months, using 0.5 L solution with 2 ml L^{-1} of rhizospheric microflora, humates or their combination and water for the control.

Microbial strains and humic substances

The isolates used in the present study were obtained from the rhizosphere of two plants. Three strains were isolated from the rhizosphere of *Cichorium intybus* (common chicory) and four strains - from *Carex acuta* (slender tufted-sedge). Four of the strains belonged to the genus *Bacillus* and the rest three - to the genus *Pseudomonas.* The microbial strains of PGPR were cultivated dynamically at a temperature of 30°C on medium containing (per litre): 25.0 g glucose, 3.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1.0 g yeast extract, 1.0 g peptone. The culture fluid from the seven strains was mixed in equal quantities. The humic substances were obtained from lignite through biotransformation by fermentation with microfungi *Trichoderma harzianum* and *T. viride*, and the coals were taken from Stanyantsi mine in Southwestern Bulgaria (Chakalov et al., 2012). The extract contained total organic C (12,9 %), humic acid (10,87%) and fulvic acid (1,22%).

Soil characteristics

Soil pH was determined according to the standard BDC-ISO 10390. The mechanical composition of soils was determined by the method of Rutkowski. The Turin`s method was used to determine the humus content, and total nitrogen content was measured according to the Kjeldahl`s method. The standardized sample preparation method ISO 22036: 2008 - Microwave acid decomposition was used for the detection of heavy metals and metalloids in the soil. The concentrations of heavy metals and arsenic (As) were determined by ICP - OEC.

Biometric parameters

Plant height, root length and chlorophyll content index (CCI) were determined after four and a half months of vegetation. Fresh weight of the aboveground biomass and roots was also measured. Dry weight was measured after drying at 80^{o}C for 1 d. Thirty plants were used to measure fresh and dry weights of the aboveground biomass and the roots. Chlorophyll content was measured with a Chlorophill Content Meter CCM-200.

Statistical analyses

The statistical analysis of some of the biometric parameters was performed with the software Stratigraphics Centurion. The normality of the datasets for each variant was preliminary affirmed by using Shapiro-Wilk test at a significance level $p<0.05$. After that one-way analysis of variances (ANOVA) was used to estimate the statistical significance (at significance level of 0.05).

RESULTS AND DISCUSSION

The soil used in the present study was taken from a soil depot of a real mining object located in the region of Srednogorie. According to the soil classification in relevance of their reaction, the soil was medium acidic (pH (H_2O) – 5.65 and pH (KCl) – 4.39) and "slightly sandy-clay". The humus and nitrogen contents were low (0.98% and 0.196%, respectively). The concentrations ($mg.kg^{-1}$) of As, Cu, Pb and Zn were as follows: 9.2, 140.0, 28.9 and 96.9, respectively. Compared to the legal framework (Art. 5 of Regulation № 3 of August 1, 2008, on the rules on the permitted content of hazardous substances in soils) the soil was considered not contaminated with heavy metals and As.

The average values of fresh and dry weights of the aboveground biomass and the roots per plant are presented in Table 1.

Fresh and dry weights of the aboveground biomass (AGB) expressed as a percentage of the corresponding controls are presented in Figs. 2 and 3, respectively.

Table 1. Biometric parameters of white clover plants.

Variants	Fresh weight, aboveground biomass [g]	Dry weight, aboveground biomass [g]	Fresh weight, roots [g]	Dry weight, roots [g]
Control	0.384	0.061	0.069	0.034
PGPR	0.395	0.074	0.072	0.038
HA	0.678	0.134	0.098	0.049
HA + PGPR	0.703	0.158	0.099	0.050

Control – water; PGPR – plant growth-promoting rhizobacteria; HA – humic acids; HA + PGPR – combination of humic acids and plant growth-promoting rhizobacteria.

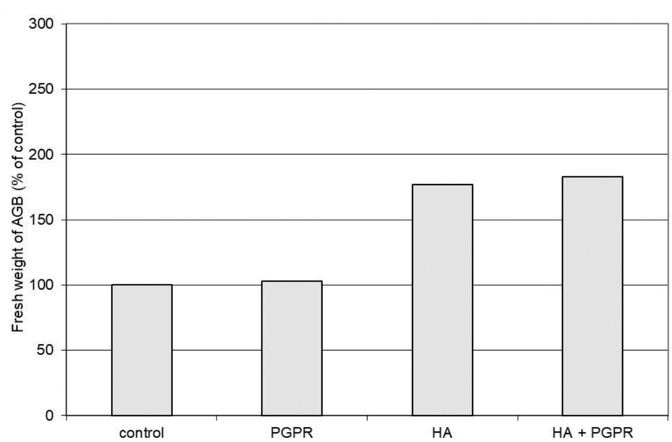

Figure 2. Fresh weight of the aboveground biomass (AGB) per plant after treatment of white clover plants with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA). Data are expressed as % of control.

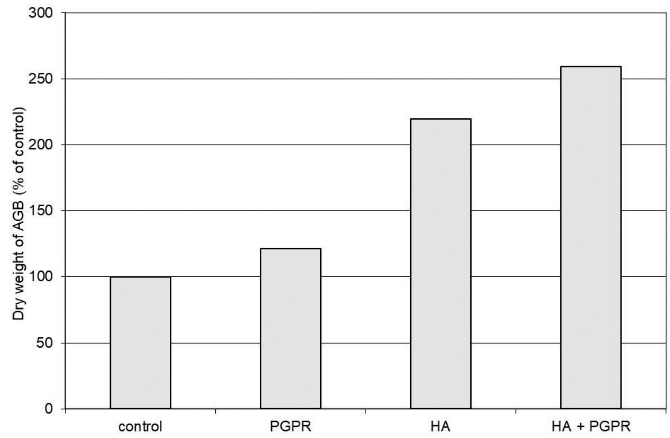

Figure 3. Dry weight of the aboveground biomass (AGB) per plant after treatment of white clover plants with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA). Data are expressed as % of control.

The results showed that treatment of white clover plants with the mixed culture of rhizospheric microflora had almost no effect on the fresh weight of the aboveground biomass (only 3% above control). However, the dry weight data indicated higher assimilation of nutrients (21% above control). As the soil was poor of humus, the application of humates had a strong positive effect (an increase of the fresh and dry weight of the aboveground biomass by 77% and 120%, respectively). The best results were obtained after the application of a combination of rhizospheric microflora and humic acids. The increase of dry biomass was by 160% compared with the control (Fig. 3).

The application of humic acids either alone or in combination with the rhizospheric bacteria increased root fresh weight by 45% compared with the control (Fig. 4). Treatment with PGPR had almost

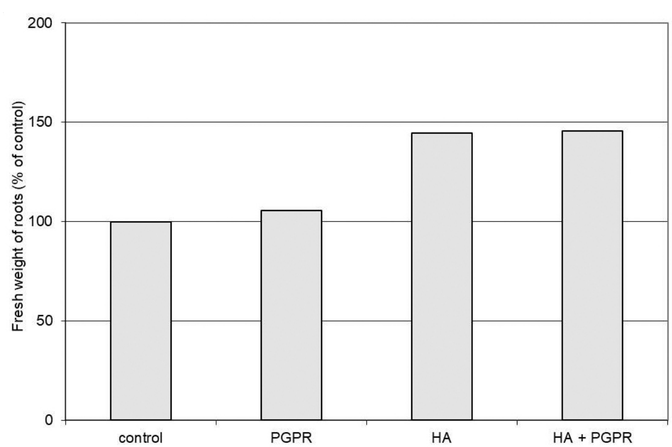

Figure 4. Fresh weight of roots per plant after treatment of white clover plants with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA). Data are expressed as % of control.

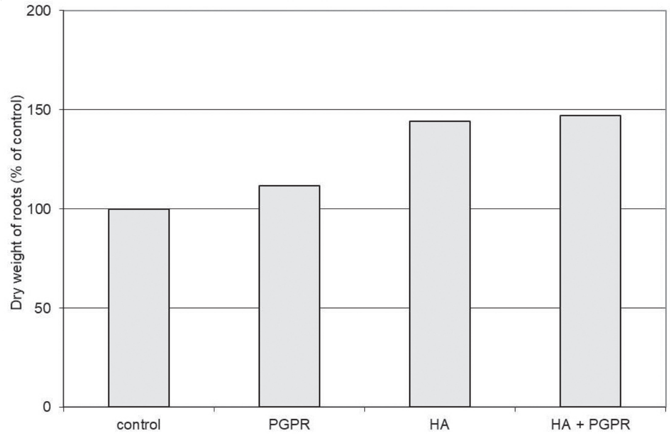

Figure 5. Dry weight of roots per plant after treatment of white clover plants with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA). Data are expressed as % of control.

no effect on root fresh weight whereas root dry weight was increased by 12% (Fig. 5). The application of humates or humates in combination with rhizospheric microflora resulted in increased dry weight of roots by 44% and 47%, respectively.

The statistical analysis of some biometric parameters (plant height, number of leaves, root length and CCI), is presented in Figs. 6–9. The box plots comprise dataset: median (line across box), mean value (small cross in the box), minimum and maximum values (lower and upper ends of the whisker, respectively), interquartile range containing 50% of values (box).

The results on plant height showed that plants treated with rhizospheric microflora were shorter compared with the control (Fig. 6), indicating that some strains could have a retardant effect on plant growth. Retardant-type growth regulators have been found to induce resistance mechanisms in conditions of water deficit, thus reducing the negative effect of water stress. The positive impact of retardants on plants is observed during both wetting and drought periods. Our results showed similar effects of treatment with either humates or the combination of humic acids and rhizospheric microflora on plant height

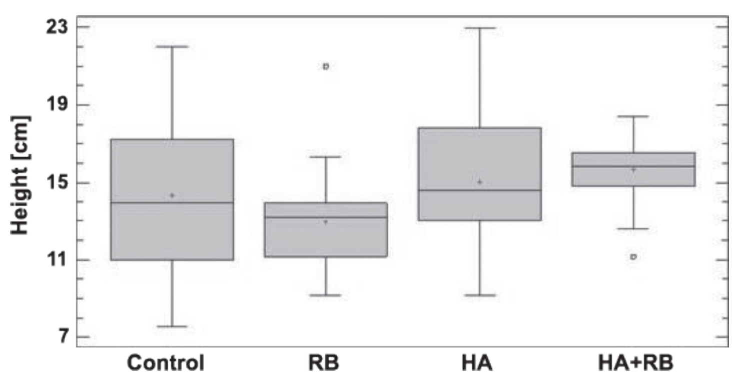

Figure 6. Plant height of white clover plants after treatment with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA).

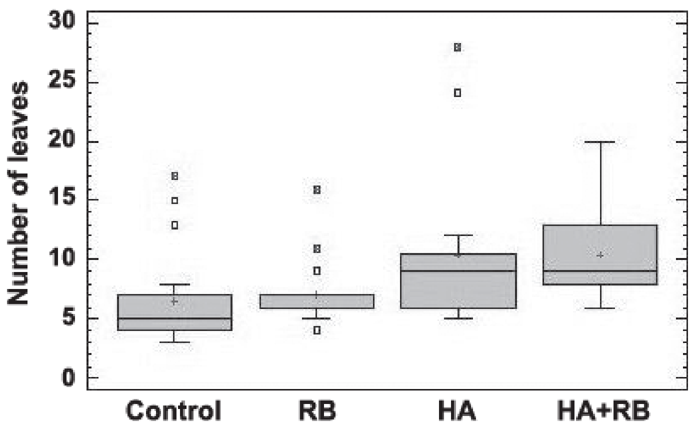

Figure 7. Number of leaves of white clover plants after treatment with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA).

(an increase by 7%, and 6% , respectively).

The three treatments applied in the present study affected the number of leaves of white clover plants (Fig. 7). The average number of leaves per plant in the control was 6.4. Treatment with PGPR, HA or their combination (PGPR + HA) increased the number of leaves to 7.2, 10.4 and 10.6, respectively.

An increase in root length of the test plants after the applied treatments was also found (Fig. 8). The average root length of the control plants was 7.2 cm. Treatment with rhizospheric microflora increased root length by 7% compared with control. The application of humic acids either separately or in combination with PGPR increased the average root length by 13%. It should be noted that plants treated with rhizospheric microflora had significantly better developed lateral roots when compared with the other treatments.

According to Liu et al. (2019) the measurement of CCI is a fast noninvasive *in vivo* method for quantitative assessment of pigmentation. The relative chlorophyll content is an appropriate indicator to be

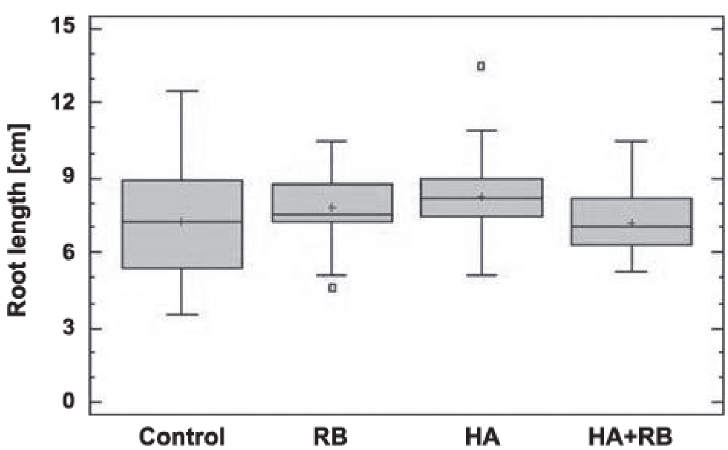

Figure 8. Root length of white clover plants after treatment with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA).

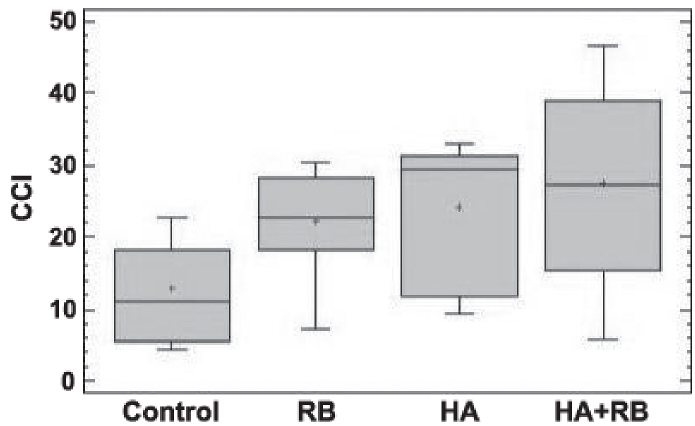

Figure 9. Chlorophyll content index (CCI) of white clover plants after treatment with rhizospheric bacteria (PGPR), humic acids (HA) and their combination (PGPR + HA).

used for biomass assessment, as CCI correlates positively with both N content and yield. In our experiments, a strong positive effect on CCI was observed in all treatments (Fig. 9). The application of PGPR alone increased CCI by 75%. The greatest effect (115% above the control) was observed after the combined application of bacterial strains and humic acids.

The statistical analysis showed that the selected threshold for significance was 0.05 (p-value). Regarding the number of leaves and CCIs there was a statistically significant difference amongst the standard deviations at the 95.0% confidence level, because the p-values were below 0.05 - <0.0001 and 0.0263, respectively. The p-values about the height of the plants and their root length were exceeding this value. There was not a statistically significant difference at the 5% significance level.

CONCLUSIONS

White clover plants growing in poor soils treated with a combination of rhizospheric microflora and humic acids demonstrated better growth, which implies improvement of their mineral nutrition. The test plants had a more compact appearance due to the retardation effect of the microbial secreted growth regulator. Thus, application of humic acids in combination with rhizospheric microorganisms belonging to the genera *Bacillus* and *Pseudomonas* can be recommended for improvement of the growth of white clover plants cultivated in poor soils.

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