

## Review

# Effects of Urea on the Nitrogen Fixing Capacity and Growth of Grain Legumes

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## ABSTRACT

The grain legumes are getting popular all over the world, either because of their high protein or oil content. It has been estimated that nitrogen fixation by these grain legumes is generally insufficient to meet the N demands of high yielding plants. This demand can be fulfilled by providing N through other sources. However, it has to be determined whether N can be used for this purpose. The effect of urea as a nitrogen source was compared with other nitrogen sources for their effects on different parameters relating to N assimilation; nodulation, nitrogen fixation and plant growth. The use of urea allowed an increased N utilization as compared to other fertilizer sources. It was apparent that the process of nitrate assimilation and N-fixation can occur simultaneously and the legumes may be benefited by both.

**Key Words:** Urea; Nitrogen fixing; Growth; Grain legumes

## INTRODUCTION

Increasing cost of production, storage and transportation of nitrogen fertilizers have stimulated biological nitrogen fixing systems. Legumes have been used by farmers in rotations and soil management practices for many centuries (Brill, 1977). Most legumes have the ability to derive their constituent nitrogen from the atmosphere by the reduction of  $N_2$  to  $NH_3$ . This reduction of nitrogen is carried out by the association between rhizobia and legume roots, in the presence of an enzyme complex nitrogenase, catalyzing the production process (Salisbury & Ross, 1978). Biological nitrogen fixation by all the different types of organisms has been estimated to contribute to the earth about 175 million metric tones of nitrogen per year. Legumes grown for grain, hay, pasture and other agricultural purposes account for almost half ( $80 \times 10^6$ ) of the annual quantity of nitrogen fixed by biological system (Anonymus, 1984). Biological processes contribute about 65% of nitrogen used in agriculture (Burriss & Roberts, 1993).

The grain legumes are getting popular all over the world, either because of their high protein or oil contents. The worldwide production of all grain legumes has increased manifold from 1948 to 1998 (Anonymus, 1998). Soybean (*Glycine max*) are the most important grain legumes in USA. Biological nitrogen fixation offers an alternative to the use of expensive ammonium based fertilizer nitrogen, but the high yielding agricultural systems are difficult to sustain solely on biological  $N_2$  fixation. So supplementation with mineral nitrogen might then be necessary for maximal yield of grain legumes (Zuberer, 1998).

Not only the amount of nitrogen applied per unit area as fertilizer, but its form is also important. Urea,  $NH_4^+$ ,  $NH_3$  and  $NO_3^-$  are the most commonly used sources of fertilizer nitrogen. Differences in the effect of these nitrogen forms on plant growth, nitrogen fixation and nodulation have been observed. Growth of peas (*Pisum sativum*) and velvetleaf (*Abutilon theophrasti*) was less with urea than with nitrate, while growth of soybeans (*Glycine max*) and wheat (*Triticum aestivum*) was equivalent with urea and nitrate (Lahav *et al.*, 1976). The inhibitory effect of nitrates on nodulation of soybeans is well-documented (DeMooy *et al.*, 1973). Both nitrate and ammonium-N sources have been found inhibitory to nitrogen fixation in comparison with urea (Vigue *et al.*, 1977). The superiority of urea is still not clear because under soil conditions urea is converted to nitrate. The subject needs to be discussed in more depth. The objectives of this paper is to discuss the effects of urea in comparison to other N sources, on nitrogen assimilation, nodulation and nitrogen fixation and overall effect on plant growth in grain legumes especially soybeans, dry beans and peas.

### Effects of urea on plant growth

Nitrogen is the most critical element of plant growth. Studies conducted on grain legumes revealed that plant growth is affected differently by various N sources (Ryle *et al.*, 1978). A comparison of different N sources showed that seedling growth (fresh or dry weight) was always more in nitrate than various reduced N sources (Lahav *et al.*, 1976). However, urea was better than other reduced N sources (glutamine, ammonium malate and other ammonium salts). But in another study, urea appeared to be the best source which gave the highest dry matter yield when four nitrogen sources

( $\text{NH}_4^+$ ,  $\text{NH}_4\text{OH}$ ,  $\text{NO}_3^-$  and urea) were compared (De Mooy *et al.*, 1973). In peas (*Pisum sativum*) it has been observed that plant allocate different relative proportion of total N contents to leaves, stems and roots (DeJong & Phillips, 1981).

a) **Leaves.** Nitrate grown plant of soybeans exhibited fully expanded second trifoliated leaves while only half the plants grown in urea had second trifoliate leaves (Lahav *et al.*, 1976). In another study on soybeans, plants given nitrate nitrogen developed branches from the axils of cotyledon, primary and subsequent trifoliate leaves (Ryle *et al.*, 1978). In addition to N form, when varying levels of N source (urea) were applied to dry beans (*Phaseolous vulgaris*) leaves responded relatively more at 140 mg urea/pot, although there was a further increase in leaf dry matter at higher urea levels: 280 mg urea/pot (unpublished data, plant physiology green house). The form of the available N affects photosynthesis by producing markedly different values of carboxylation efficiency and  $\text{CO}_2$  exchange rate. The leaves of plants treated with  $\text{NO}_3^-$  and  $\text{NH}_4^+$  showed lower values of carboxylation efficiency and  $\text{CO}_2$  exchange as compared to leaves on plants relying wholly on nitrogen fixing symbionts (Bethlenfalvay & Phillips, 1978). There could be competition for electron between  $\text{CO}_2$  and  $\text{NO}_2$  reduction mechanisms in the chloroplast. The low  $\text{CO}_2$  exchange rate in  $\text{NH}_4^+$  treated plant might probably be due to increased  $\text{NH}_4^+$  influx and accumulation to toxic levels in the leaves. Protein synthesis in young leaves is higher and these same young leaves are also very active in  $\text{NO}_3^-$  reduction (Pate, 1968). The synthesis of enzyme (nitrate reductase) in leaves is enhanced with the application of nitrate because nitrate is translocated to the leaf cells more rapidly (Salisbury & Ross, 1978). The N contents of leaves is rapidly converted to protein and during seed development leaf N is transferred to seed for protein production (Strecker, 1972).

b) **Stem.** It has been reported that in the early stages of growth of soybeans (3 weeks after planting) nitrate comprise about 40% of the N in the stem. It was further noted that during earliest growth stages of soybean, over 1/4<sup>th</sup> of the N in the stems plus petiole was in the form of asparagine, suggesting that this compound is a temporary storage form of nitrogen in stem (Strecker, 1972). In this study it was also observed that the proportion of nitrate (40%) decreased rapidly to around 5%. Effects of different N forms on the stem dry weight and N content were not significant (Rabie *et al.*, 1979). There is a positive correlation between branches, yield and main stem yield, which increased as the N inputs increased (Andrew *et al.*, 1981). The stem dry weight of dry bean plants increased constantly with high (280 mg urea/pot) rates of urea, while this increase in leaves and

root dry weight at this urea level was negligible (unpublished data, plant physiology green house). This might be due to enhanced accumulation of assimilates in the stem at this level. The N content of the stem might also be higher as reported by DeJong and Phillips (1981) who in their experiment on peas found that the relative amount of N allocated to the stem increases with the highest level of  $\text{N}_2$  fixed.

c) **Roots.** A comparison of different N forms showed that nitrate treatment were slightly more promotive on the dry weight of the root system than ammonium and urea (Rabie *et al.*, 1979). Application of either N or P had no effect on root branching, but N fertilization in the presence of an adequate P supply enhanced growth hormone synthesis in the roots of soybean and increased branching of the root system (De Mooy *et al.*, 1973). High level of N application (280 mg urea/pot) to dry bean plants do not show any positive effect on root dry weight. This might be due to limited space for root development in pots (unpublished data, plant physiology green house). The nitrate contents of roots of soybean plant grown in nutrient solution containing nitrate was generally approximately the same as the nitrate content of the leaves and lower than the nitrate contents of stem (Strecker, 1972). The relative amount of N in the peas decreased with the increase in  $\text{N}_2$  fixation (DeJong & Phillips, 1981).

d) **Dry matter accumulation and seed yield.** Growth response of soybean plants cultured in nutrient solutions containing varying concentration of urea or  $\text{NO}_3^-$ -N were observed by Vague *et al.* (1977) The total dry matter accumulation was less at three and six mM urea N than 3 mM  $\text{NO}_3^-$  and 6 mM  $\text{NO}_3^-$  respectively. At 12 and 19mM N levels, there was no difference in dry matter accumulation between N source. In a field study the application of nitrogen to mungbean increased the seed yield which was associated with increase in pod number per plant and seed number per pod (Hamid, 1991). With a dose 10-20 kg  $\text{Nha}^{-1}$  there is similar significant increase in seed yield in green gram and black gram (Nagre, 1982). In another study, Lahav *et al.* (1976) reported that the total dry matter production at 3 mM urea was equivalent to that of plants grown in 12 mM  $\text{NO}_3^-$  and was maximal with 6 mM urea (12mM N) in the presence of 50 gm resins/liter. Results obtained with N fertilization of soybeans in the field are extremely variable and this could be due to paradoxical interaction between mineral (soil and fertilizer) nitrogen and symbiotically fixed nitrogen and other factors such as temperature, rainfall and method of fertilizer application. Comparing different nitrogen forms and method of application, it was observed that except for the surface application of ammonium which increased both the seed

yield and pod number per plant by 12 and 21%, respectively, the other N treatments were either not effective or depressive. The dropping percentage with N treatments ranged from 12-46% while there was no dropping in the control (Rabie *et al.*, 1979). Application of N @60 kg N ha<sup>-1</sup> increased nodule formation and dry weight per plant in mungbean (Othman & Ismail, 1987). It has been found that seed yields of mung bean and urd beans were increased by increasing N rates from 0 to 10 and 20 kg/ha (Yadav *et al.*, 1990). In dry beans it has been reported that total nitrogen uptake were related to the seed yield of different dry bean cultivars (Westermann & Kolar, 1978).

#### **Effect of urea on nodulation**

Infection of legumes by the rhizobia usually, but not invariably, takes place through the root hairs. In the presence of rhizobia root hair branching and curling occurs. Entry to the root hair is frequently at the point of curling. The bacteria are thought to penetrate the primary cell wall than cause invagination of the inner cell wall and plasmalemma leading the inward going "infection thread". This thread then advances to the cortical cells of the root where cell division occurs in the inner cortex into the bulbous enlargement: the root nodule. The nodules consist of enlarged plant cells most of which are packed with bacteria. Nodules with a red or pink region usually are active in N<sub>2</sub> fixation and are said to be effective, whereas nodules which are white or greenish brown, are ineffective and said to be senescing. Trung and Yoshida (1983) grew legumes in nutrient solution containing 0-100 ppm N in the form of urea and found that maximum plant height, number of nodules and fresh weight of tops and roots were obtained at 25 ppm nitrogen.

**A. Nodulation response to Rhizobia strains.** It has been found that Rhizobial strain had a finite host range, nodulating certain legumes but not others. This led to the concept of cross-inoculation, with legumes grouped according to different rhizobia with which they formed nodules. The rhizobia isolated from the species of *Medicago* will not nodulate *Melilotus*, and vice versa. More than 20 cross-inoculation groups have been identified up till now (Graham, 1998). This specificity occurs at each stage of nodulation process. In addition to host specificity, different strains differ in their ability to induce nodulation and fix nitrogen as varieties differ in their susceptibility to nodulation. In the case of soybean, it has been found that a specific *Rhizobium japonicum* strain which is capable of inducing nodulation and fixing nitrogen in some varieties, failed to induce nodulation on genotypes, where nitrogen is fixed by other *Rhizobium japonicum* strain (Vest *et al.*, 1973). In extensive greenhouse experiments on the effects of *Rhizobium* strains,

host genotypes, and plant nutrition on symbiotic nitrogen fixation, it was concluded that the response of dry beans (*Phaseolus vulgaris*) to rhizobia varies widely with host genotype and with nutritional factors (Burton, 1976).

**B. Combined Nitrogen effects.** It has been reported that nodulation is greatly reduced by higher application of nitrogen irrespective of nitrogen source (Harper & Cooper, 1971; Franco *et al.*, 1979). Nodule number and size are usually reduced when the supply of soil or fertilizer nitrogen is increased. It has been reported by DeMooy *et al.* (1973) that nodule number was reduced in soybeans with all rates of N fertilization in pot trials. They further pointed out that nodule numbers were reduced by about 33%, fresh weight by 50% and nodule size by 25% when 168 kg ha<sup>-1</sup> N was applied and a stronger reduction occurred when 672 kg ha<sup>-1</sup> N was applied on soil that had part of its available nitrogen immobilized by incorporation of corn cobs. Different nitrogen sources have shown varying effects on nodulation. In an experiment it was found that total nitrogen uptake varied little with N rate, but as the N fertilizer rate increased (above 300 kg N ha<sup>-1</sup>) the proportion of N produced by fixation decreased (Borin *et al.*, 1992). Criswell and Hume (1972) showed in their comparison of anhydrous ammonia and organic matter that NH<sub>3</sub>-N reduced nitrogen fixed per hectare by 39 to 48% below control plots by reducing nodule mass in the presence of deep placed NH<sub>3</sub>-N. However, the addition of organic matter was successful. In a study on peas (*Pisum sativum*) it was noted that the nodule mass declined with nutrient containing more than 2 mM NH<sub>4</sub><sup>+</sup>. The nodule numbers per plant remained stable in plants given nutrient solution containing upto 8 mM NH<sub>4</sub><sup>+</sup> and declined at 16 mM NH<sub>4</sub><sup>+</sup> (Bethlenfalvay *et al.*, 1977). Cartwright (1967) in his studies noted that the effects of urea on nodule number were in general smaller than those of similar amounts of nitrogen added as nitrate to the mineral salt medium. When he supplied both sources (urea and nitrate) in rooting medium, nitrate showed greater effect than urea, but when they were fed to the cut basal end of the excised roots nitrate only slightly delayed nodulation while urea inhibited it almost to the same extent as when supplied in the rooting medium. The conclusion by Harper and Cooper (1971) that urea appear quite attractive as a N source because it allows extensive nodulation relative to nitrate, needs further investigation because as surface application of NO<sub>3</sub><sup>-</sup> was the most promotive to nodule dry weight and basal dressing of ammonium or urea was slightly promotive, while urea spray was repressive.

#### **Nitrogen assimilation**

**A) Urea as a nitrogen source.** Urea is a dominant source in many areas of the tropics. It has some obvious

advantages in terms of high analysis, relatively low raw material cost, non-explosive character and lower acidifying properties than ammonium sulfate (Engelstand & Russell, 1975). Farmers and agricultural scientists have found that the response of crops to the nitrogen in urea compares favorably with the nitrogen in  $\text{NH}_4\text{OH}$  (Lahav *et al.*, 1976). Urea converts rapidly to ammonia in the soil by hydrolysis. If applied properly, urea is as effective as other N sources in common use. Volatilization losses occurring from top dressing of most ammonium-N sources were much greater with heavier rates of urea used as top dressing while incorporation of urea in the soil reduced volatilization losses (Engelstand & Russell, 1975). With some species yields obtained with urea were as good or better than those with  $\text{NO}_3^-$ , however urea uptake by the soybean plants was found to be slower than  $\text{NO}_3^-$  regardless of urea concentration supplied (Vigue *et al.*, 1977).

**B) Nitrate Reductase.** Nitrate reductase (NR) is the first enzyme in the pathway of nitrate assimilation and plays a key role in the regulation of the assimilatory reduction of nitrate. Nitrate reductase is a flavoprotein of high molecular weight, varying from 230,000 to 500,000 depending on the organisms in which it occurs. It contains several prosthetic groups such as FAD, cytochrome b557 and Molybdenum. One of the well documented features of this enzyme is that it can exist in two stable forms i.e one active and another inactive, and that their mutual inter-conversion is governed by an oxidation reduction mechanism. This inter-conversion may have a great significance in plant productivity regulating the ratio of carbohydrate to protein (Aparicio & Maldonado, 1977).

#### **Nitrate reductase as a criterion for the selection of superior cultivars and predictive test for yield**

The attempt to establish the activity of NR as a criterion for the selection of superior cultivars has been variable. Hegeman *et al.* (1977) in their investigation with 14 sets of corn hybrids observed that on an average only 15% of the variation of the grain-N and yield was related to the activity of NR. However the data did establish: a) That the enzyme was heritable (general and specific combining abilities were significant), b) That selection for NR activity would be effective in a synthetic cultivar made from these inbred and c) that two of the inbreds would be effective tester lines in a selection program for NR activity. Johnson *et al.* (1976) in their experiments with wheat and NR activity concluded that although the nitrate reductase measurements do not correlate significantly with the final yield, yet they provide a useful test for future yield, except at the highest nitrogen levels.

**C) Nitrate reductase activities.** Shortly after germination there is a measurable level of nitrate reductase in both roots and shoots. As the plant matures, the activity in roots disappear and the upper canopy apparently takes over most of the reduction (Oakes, 1977). It has been confirmed from other studies that only very low amounts of nitrate reductase activity was found in roots, with much more activity in the leaves (Dale, 1977). Nitrate is both an inducer and substrate for NR, application of 5 mM  $\text{KNO}_3$  to the *Phaseolous vulgaris* plants grown at different urea concentrations caused induction of nitrate reductase activity (unpublished data, plant physiology green house). The NR activity with the highest urea concentration (280 mM urea per pot) was only double (0.777  $\mu\text{mol. NO}_2/\text{g fresh weight/h}$ ) than that of the control (0.35 U mol  $\text{NO}_2/\text{g fresh weight/h}$ ). It has been shown that the patterns of induction of the enzyme were coincident with the patterns of nitrate uptake (with respect to time and external supply of nitrate). This is consistent with the concept that the nitrate flux to the leaves from the roots plays a much larger regulatory role than the leaf nitrate content in controlling the NR activity in the intact plants (Hegeman, 1977). This reveals that it is almost impossible to substitute nitrate by ammonium or urea in terms of NR activities.

#### **Nitrogen fixation**

**A) Nitrogenase activities.** The essential components for symbiotic  $\text{N}_2$ -fixation are  $\text{N}_2$  (electron acceptor) and the enzyme nitrogenase. All the nitrogen fixing organisms contain an enzyme complex called nitrogenase, which does not seem to vary significantly in structure from one species to another. No organisms that lack this enzyme complex, are able to fix nitrogen. The nitrogenase system is a complex of two proteins, neither of which has any demonstrable activity by itself, one is a molybdenum-iron sulfur protein (Mo-Fe protein) which is known as dinitrogenase. It contains two atoms of molybdenum, 22-24 of iron and 25 to 30 of acid labile sulfur. The other component is an iron protein (Fe-protein) designated as dinitrogenase reductase. Nitrogenase, besides reducing  $\text{N}_2$  to  $\text{NH}_3$ , is capable of reducing acetylene to ethylene. Ethylene is measured by gas chromatography and is being used as a measure of nitrogenase activity (Zuberer, 1998).

**A.1) Nitrogen source effects on nitrogenase activities.** Continuous application of fertilizer nitrogen reduces nitrogenase activities (Franco *et al.*, 1979) but it has been suggested that low levels of nitrogen promote symbiotic  $\text{N}_2$  fixation in legumes (Dart & Wildon, 1970; Bethlenfalvay *et al.*, 1978) or do not affect nitrogenase activity (Franco *et al.*, 1979). The mechanism of this phenomenon might be explained as an enhancement of

photosynthesis at a low N level (2mM  $\text{NH}_4^+$ ) as  $(\text{NH}_4)_2\text{CO}_3$ ) due to relief on N stress, but without the inhibition of  $\text{N}_2$  fixation caused by high levels of combined N. The effect of 17 ppm ammonium-N or nitrate on different stages of nodule formation and on the formation of nitrogenase complexes in the *Rhizobium-Melilotus/Medicago sativa* symbiosis were investigated. Ammonium-N addition did not reduce the nitrogenase activity and nitrate-N stimulated N-fixation by root nodules (Vernichenko & Mishustin, 1990). In a green house study with dry beans (*Phaseolous vulgaris*) different levels of urea (28,56,140, and 280 mg urea/pot) depressed nitrogenase activity when compared with plants having no urea treatment, however the depressive effect at the low urea level (28 mg/pot) was much less than at the highest level (280 mg/pot) (unpublished data, plant physiology green house, plant physiology green house). Although 50% reduction in nodule weight occurred by urea application, the amount of atmospheric  $\text{N}_2$ -fixed was only slightly reduced (DeMooy *et al.*, 1973). Comparing ammonia and nitrate, Gibson (1974), reported that ammonia caused less inhibition of  $\text{N}_2$ -fixation than nitrate in hydroponic study with soybeans compared with urea and nitrate. They found consistently a higher percentage contribution of  $\text{N}_2$ -fixation by nodule with urea versus  $\text{NO}_3^-$  grown soybeans plants. It has been almost confirmed that  $\text{NO}_3^-$  is more inhibitory to nodule formation and function than urea (Harper & Nicholas, 1976).

**A.2) Hydrogen evolution and hydrogenase activity.** During  $\text{N}_2$ -fixation nitrogenase also produces  $\text{H}_2$  as a by product and  $\text{H}_2$  production represents a loss of energy that otherwise would be available for  $\text{N}_2$  fixation. Estimates of losses of energy from many agricultural legumes have ranged between 20 and 40% of the electron flow through nitrogenase. There is evidence that environmental conditions such as temperature, growth stage, and light intensity affect  $\text{H}_2$  evolution from nodules (Evans *et al.*, 1980). It has been reported that some rhizobial bacterioids possess two enzymes catalyzed reactions that participate in  $\text{H}_2$  metabolism. These include the ATP dependent  $\text{H}_2$  evolution reactions of nitrogenase and uni-directional hydrogenase which catalyses  $\text{H}_2$  oxidation. Some rhizobium strains synthesize a unidirectional hydrogenase system in legume nodule bacterioids. This system participates in the recycling of hydrogen that otherwise would be lost as a by product of the nitrogen fixation process (Albrecht *et al.*, 1979). The physiological role of  $\text{H}_2$  evolution during  $\text{N}_2$ -fixation is unknown. Possibly  $\text{H}_2$  is used by hydrogenase to react with free  $\text{O}_2$  to maintain an anaerobic environment for nitrogenase (Vest *et al.*, 1973).

## **B) Energy requirement for nitrogen fixation.**

Biological nitrogen fixation requires large inputs of metabolic energy i.e. the ATP for conversion of  $\text{N}_2$  to  $\text{NH}_3$ . This energy is obtained from products of photosynthesis of host plant (Vest *et al.*, 1973). Sucrose, glucose and organic acids are translocated to roots and nodules and the oxidation of these substrates within the bacterioids generates the essential energy. It has been reported that number of molecules of ATP required to reduce nitrate is the same as that required to fix the same amount of nitrogen if the biological pathway is considered alone (Boulter, 1977). However, in addition to the energy costs of the biochemical pathway, energy is also required to assemble and maintain the necessary physiological environment including the production of nodules, etc. Furthermore, nitrogen fixation which essentially takes place in the roots, requires carbohydrate transport to roots from leaves and this requires energy.

## **CONCLUSIONS**

A. Urea appear quite attractive as a N source as it allows extensive legume nodulation relative to  $\text{NO}_3^-$ . The use of urea may allow increased N utilization from fertilizer source without a concomitant decrease in symbiotic  $\text{N}_2$  fixation, providing that inter conversion of urea to  $\text{NO}_3^-$  under field conditions can be inhibited. However, urea uptake was slower than  $\text{NO}_3^-$  uptake.

B. The enzyme nitrate reductase exists in urea grown plants, but is not likely to contribute much to the metabolism of the plant due to low substrate levels. But it may immediately assimilate nitrate after it is supplied to the plant.

C. It appears that the process of nitrate assimilation and  $\text{N}_2$ -fixation can occur simultaneously and the legumes may benefit from both. In dry beans (*Phaseolous vulgaris*) the maximal nitrogenase activity occurs at flowering. The activity of soybean root nodules has a reciprocal relationship to that of nitrate reductase. The maximum levels of nitrogen fixation occur at early pod fill stage, when NR activity dropped to 25% of maximum.

D. The differences in cultivars and rhizobia strains in their nitrogen fixing capacity, hydrogen metabolism and grain yields indicate that it may be possible to enhance symbiotic  $\text{N}_2$ -fixation, reduce  $\text{H}_2$  evolution and increase grain yields through plant breeding. This may reduce the need for combined N source as urea or  $\text{NO}_3^-$ .

E. The legumes are self sufficient for nitrogen requirements derived from symbiotic nitrogen. However, maximal growth and yield may depend on supplementary nitrogen, especially during early stages of

vegetative growth, and in the case of *Phaseolous vulgaris* during reproductive growth as well.

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**(Received 29 July 2000; Accepted 08 September 2000)**