Summary

Agriculture in modern times is getting more and more dependent upon the steady supply of artificial fertilizers and pesticides with the introduction of green revolution technologies. It was only after World War II that chemical pesticides and other agrochemicals were widely adopted as part of the "green revolution" in India. Chemical pesticides had many advantages, and became an integral part (along with chemical fertilizers, mechanization, and high-yielding crop varieties) of modern agriculture. A large proportion of the productivity increased during the "green revolution" was due to the widespread use of new pesticides. Often these agrochemicals were applied on a routine or prophylactic basis, resulting in immediate yield increase but contributing to future environmental, agricultural, and socio-political problems. The United Nations Food and Agriculture Organization (FAO) estimated that the total demands for agricultural products will be 60 per cent higher in 2030 than present time and more than 85% of this additional demand will come from developing countries. For over half a century, the world has relied on increasing crop yields to supply an ever increasing demand for food due to population growth. World cereal production increased significantly during last three decades due to heavy use of fertilizers and pesticides. Continued use of huge amounts of different kinds of agricultural pesticides increases their concentration in the organism and further multiplies through food chain and a phenomenon called biomagnifications. The pesticides move up in the food chain and affect the apex species in the food pyramid. Man is also situated at the higher trophic
level of food accumulates these poisons and many cases of food poisoning and contamination are recorded.

Rice (*Oryza sativa* L.), the most important food crop in the developing world and a staple food feeds nearly half of the planet’s inhabitant. More than 90% of the world’s rice is grown and consumed in Asia where 60% of the earth’s people live. Unfortunately, just like any other crop, the rice production system is also infested by a host of pests— insects, weeds, diseases, rodents, etc. They exact a heavy toll on crop production efforts. To solve these pest problems, pesticides have been resorted and it appears that their use will continue, at least in the near future, notwithstanding increased interest in integrated pest management; furthermore, integrated pest management does not preclude pesticide usage.

Any chemical that can kill or repel a pest is known as chemical pesticide. Pesticides are often used in agriculture to protect crop plants from competition with abundant but unwanted other plants species also known as weeds. The need of weed management is as old as agriculture itself. Pesticides, falling into three major classes: insecticides, fungicides, and herbicides (or weed killers), are required. Herbicides, specifically, are used for control of weeds. Herbicides are the most widely used class of pesticides accounting for more than 60% of all pesticides applied in agriculture. The intention of any herbicide treatment is to reduce the abundance of weeds to below some economically acceptable threshold, judged on the basis of the amount of damage that can be tolerated to crops. Sometimes, this objective can be attained causing significant damage to non-target plants. This is especially true for herbicides, because they are toxic to a wide variety of plant species, and just the non target organism like biofertilizer. Bio-fertilizers are eco-friendly fertilizers, which are being used to improve the quality and fertility of the soil. The main sources of bio-fertilizers are bacteria, fungi and cyanobacteria (blue-green algae). Bio-fertilizers are being viewed as the future of fertilizers, as they have the ability to solve the problems of salinity of the
soil, chemical-run offs from the fields. They, therefore, ensure the well being of the nutrients present in the soil, therefore making the soil more fertile with time. Nutrient need of growing plant can be met through a number of sources. The major sources of plant nutrient are minerals fertilizer, organic manure, recycled waste and byproduct, biological nitrogen fixation (BNF), natural minerals and to lesser extent nutrient recycled through irrigation water and precipitation. In recent years, biofertilizer containing micro-organism such as symbiotic system- *Rhizobium, Frankia, Azolla* etc. non-symbiotic system-*Azotobacter, Azospirillum*, blue green algae etc. Therefore, the broadcast spraying of herbicides results in broad exposures of non-pest species, which can cause an unintended but substantial mortality of these biofertilizers. In many cases, the herbicide toxicity has to led interfere with biochemical pathways, such as photosynthesis, essential amino acids biosynthesis and chlorophyll biosynthesis. However, the import of various herbicides to the system results in the abnormal biochemical/physiological metabolisms in plants. Herbicides cause toxicity and cellular disruption in plant species through induction of oxidative stress via increased generation of active oxygen species (AOS). Hence, excess of herbicides can cause oxidative stress due to increased production of AOS and subsequently accelerated peroxidation of lipids and oxidation of proteins. Herbicides can also interfere with nitrogen metabolism and antioxidative capacity of plants. As antioxidants are free radical scavengers, they protect plants from various factors through tolerance mechanism. Tolerance can be achieved if plant metabolism and cellular integrity are maintained by balancing the redox state of the cell. The major basis for the tolerance of crops to most herbicides is differential rates and routes of herbicide metabolism. In many plants, the selection pressure of pesticide toxicity led to the natural evolutionary development of tolerant plant genotypes in response to agrochemical pollutants. Many plant species are known to either possess or lack the genetic variability for tolerance of agrochemicals.
Another anthropogenic related problem is increase in chlorofluorocarbons in the atmosphere which depletes the earth’s stratospheric ozone layer, and a decrease in the ozone column has led to an increase in levels of ultraviolet-B (UV-B: 280–320 nm) radiation that reach the earth’s surface. Ozone depletion in the stratosphere has resulted in increased ultraviolet B radiation at the earth’s surface since the 1980s and present projections estimate a return to pre-1980 levels by 2050–2075. In addition, stratospheric ozone recovery may possibly be delayed due to a number of uncertainties. Therefore, it is still essential to investigate the effects of enhanced UV-B radiation on various aspects of plant metabolism. Enhanced UV-B radiation can affect many aspects of the plant growth and metabolism. UV-B radiation has been shown to affect plants from the molecular to the ecosystem level and multiple target sites for UV-B action have been known. Plants species that are sensitive to UV-B radiation often exhibit changes in morphological traits, e.g., reduction in height, biomass accumulation and leaf area and physiological properties. Adverse plant responses to enhanced UV-B depend on the level and duration of the stress. Extensive studies on the effect of UV-B radiation on plant physiology, growth and development have shown that there are interspecific and intraspecific differences at the levels of UV-B tolerance, which differ considerably between genera, species and even closely related species, and even other factors. However, many plants are quite resistant to UV-B radiation and possess a number of UV protection mechanisms. One of the most important mechanisms is screening out UV-B radiation by accumulation of flavonoids or other UV-absorbing compounds in the leaf epidermis. Other mechanisms that have received less attention than epidermal screening mechanisms are enzymatic and non-enzymatic antioxidant defense systems that may mitigate UV-induced damage that occurs due to the production of active oxygen species (AOS). The AOS potentially induced by UV-B radiation can cause oxidative damage to membrane lipids, nucleic acids, and proteins. To keep this damage to a
minimum, plants possess enzymatic and nonenzymatic antioxidantive defense systems.

Pretilachlor (chloroacetanilide), a selective systemic herbicide, commonly used in the rice field, is widely used as herbicide to control grasses, sedges, broad leaved weeds like *Echinochloa, Cyperus iria, Cyperus difformis, Fimbristylis milliaceae, Ludwigia parviflora, Pannicum repens* etc. in rice fields particularly in Asian and South American countries. Nowadays, environmental problems are multiple and complex, especially those arising from the identification and the assessment of the toxicity of such substances. Although, several studies have demonstrated individually, the effect of rice field herbicides and enhanced UV-B radiation on plants, algae, cyanobacteria etc. No information is available regarding the tolerance and toxic effect of these pesticides on the locally available biofertilizer *Azolla* in paddy field. Similarly, due to obligatory light dependency of *Azolla*, little is known regarding the harmful effects of enhanced UV-B radiation on this plant. However, in spite of the vital role of *Azolla* as biofertilizer in rice cultivation, especially in tropical countries, little attention has been paid to understand the impact of herbicide and enhanced UV-B radiation on its metabolism. In the present investigation, we have made an attempt to assess the impact of pretilachlor and enhanced levels of UV-B radiation separately as well as in combination on physiological and biochemical activities of *Azolla microphylla* and *Azolla pinnata*, also to determine whether one type of stress affects the response of organism to other stress.

In the present study, biomass accumulation and total chlorophyll contents were analysed to select the required doses of herbicide pretilachlor (manufactured by Krishi Rasayan Exports Pvt. Ltd., J & K) from 2, 5, 10, 15, 20, 25, 30, 40 and 50 µg ml\(^{-1}\) and enhanced levels of UV-B from 1.1, 2.2, 3.3, 4.4, 5.5 and 6.6 kJ m\(^{-2}\) day\(^{-1}\) (above ambient UV-B; 8.6 kJ m\(^{-2}\) day\(^{-1}\) radiation) in the screening experiments. Out of these doses 5, 10 and 20 µg ml\(^{-1}\) of pretilachlor, which correspond to LD-5, LD-10 and LD-20 for *Azolla microphylla* and LD-15,
LD-20 and LD-30 for *Azolla pinnata*, and low (UV-B1: ambient + 2.2 kJ m\(^{-2}\) day\(^{-1}\)) and high (UV-B2: ambient + 4.4 kJ m\(^{-2}\) day\(^{-1}\)) doses of enhanced UV-B radiation, which correspond LD-5 and LD-10 for *Azolla microphylla* and LD-10 and LD-20 for *Azolla pinnata*, were considered for detailed study. The growth pattern of the *Azolla microphylla* and *Azolla pinnata* was analysed by observing the biomass accumulation, relative growth rate and protein content. Biomass accumulation (fresh and dry mass) of both species declined under 5, 10 and 20 µg ml\(^{-1}\) of pretilachlor treatment. Enhanced levels of UV-B radiation also showed considerable reduction in biomass accumulation, which further declined under combined doses of pretilachlor and UV-B. Similar pattern in the reduction of protein content in both species was also observed. Thus, the study revealed that overall growth declined with rising concentration of pesticide and enhanced UV-B doses applied separately and in combination. Though, both species of *Azolla* showed appreciable damaging effect due to pretilachlor and enhanced UV-B radiation, but compared to *Azolla pinnata*, *Azolla microphylla* exhibited greater resistant against tested stress.

The photosynthetic pigments, chlorophyll \(a\) and \(b\) and carotenoids contents decreased with increasing doses of pretilachlor and UV-B. Among photosynthetic pigments, chlorophyll \(b\) was found to be more affected in both species than chlorophyll \(a\) and the effect was greater in *Azolla pinnata* rather than *Azolla microphylla*. The greater effect on chlorophyll \(b\) can also be visualized by greater Chl \(a\) : Chl \(b\) ratio with rising doses of pretilachlor and UV-B alone and also in combinations in both species of *Azolla*. *Azolla pinnata* showed comparatively more reduction in photosynthetic pigments than *Azolla microphylla*. Thus, the study demonstrated that *Azolla pinnata* was more sensitive to both stresses pretilachlor and UV-B. The least effect was noticed on carotenoids content.

Besides photosynthetic pigments, the primary photosynthetic reactions are considered to be the direct target of herbicides and enhanced UV-B radiation. Photosynthetic oxygen yield and photosynthetic electron transport
activities i.e. photosystem II (PSII), photosystem I (PSI) and whole chain photosynthetic reaction of Azolla microphylla and Azolla pinnata exposed to pretilachlor and UV-B stress alone and in combination were analyzed. The fronds of Azolla microphylla and Azolla pinnata showed variation in photosynthesis (oxygen production). The study illustrates that the application of the pretilachlor and enhanced UV-B doses reduced the photosynthetic oxygen yield i.e. rate of oxygen production decreased with increasing dose of pretilachlor and UV-B. Pretilachlor at 5, 10 and 20 µg ml⁻¹ when combined with UV-B_1 or UV-B_2 doses resulting further decrease in photosynthetic oxygen yield in both species. The results also depict that inhibitory effect on photosynthetic oxygen yield in Azolla pinnata was even more than Azolla microphylla.

To understand the possible mechanism of action of tested stress on photosynthetic components of light reaction, PSII, PSI and whole chain reactions were analysed in the Azolla fronds already exposed to pretilachlor and UV-B radiation singly and in combination. PSII, PSI and Whole chain electron transport activities were inhibited at tested doses (5, 10 and 20 µg ml⁻¹) of pretilachlor and enhanced UV-B radiation (UV-B_1 and UV-B_2) in Azolla microphylla as well as in Azolla pinnata, and the inhibition was found to be concentration dependent. Compared to PSI activity, PSII and whole chain activities were strongly inhibited in both species, and inhibition was comparatively greater in whole chain activity than PSII activity under pretilachlor and enhanced UV-B radiation. The inhibition of the whole chain electron transport activity was all the time higher than that of PSII in the chloroplasts of both the species. PSI activity under stress was least affected.

To study the action site of these stresses on the oxidation/reduction side of PSII reaction centre, PSII mediated DCPIP (2, 6- dichlorophenol indophenols) photoreduction was also studied. Like PSII using electron accepter p-BQ, DCPIP-Hill activity was markedly inhibited by pretilachlor as well as enhanced UV-B radiation applied separately and in combination. The
results clearly demonstrated that artificial electron donors DPC, NH$_2$OH and MnCl$_2$ could not be able to restore the activity completely indicating a heavy damage on oxygen evolving complex and oxidation side of PSII following pretilachlor and enhanced UV-B radiation. The DPC restored PSII activity greatly followed by NH$_2$OH and MnCl$_2$. The extent of restoration of PSII activity with MnCl$_2$ was observed only in *Azolla microphylla*, whereas in *Azolla pinnata* less restoration in PSII activity was observed. Overall results suggest that pretilachlor and UV-B doses alone inhibited the electron flow on oxidation side of PSII by causing damaging effect on oxygen evolving complex. Further, the damaging site was extended to PSII reaction centre and also to the reducing side when stresses were applied in combination. A significant enhancement in respiration was also obtained in fronds of *Azolla microphylla* and *Azolla pinnata* following UV-B and pesticide treatment, hence indicated strong damaging effect.

The nitrogen assimilation and photosynthesis are fundamental processes for plant productivity, because this element together with oxygen and hydrogen are main constituent of biological macromolecules. Obviously, nitrogen influences all levels of plant function, from metabolism to resource allocation, growth, and development. Thus, in the present study, we also investigated the activities of the key enzymes associated with nitrogen metabolism (nitrate assimilation and ammonia assimilation) which may provide more valuable information to clarify the effect of herbicide and enhanced UV-B on nitrogen metabolism in *Azolla microphylla* and *Azolla pinnata*. The study revealed that nitrate (NR) and nitrite reductase (NiR) activities were inhibited significantly under herbicide and enhanced UV-B radiation treatments. Although pretilachlor and enhanced levels of UV-B radiation, together exhibited further inhibition in NR and NiR activities; however the inhibitory effect of pretilachlor and UV-B alone was significantly low as compared to their combined effects.
Together with nitrate assimilation a number of other enzymes play key roles in maintaining the balance of carbon and nitrogen within plant cells. Among them are GS, GOGAT and GDH, which return the carbon in amino acids back into reactions of carbon metabolism by successive reactions. Pretilachlor and enhanced levels of UV-B radiation decreased GS and NADH–GOGAT activities in *Azolla microphylla* and *Azolla pinnata*; however, the activities were less affected in *Azolla microphylla* than that of *Azolla pinnata*. In contrast to GS and GOGAT activities, an enhancement in aminating activity of GDH in both *Azolla* species proves the role of GDH in ammonia assimilation which declined considerably due to decreased GS and GOGAT activities. Thus, under pesticide and UV-B stress, decrease in GS and GOGAT activities may disturb carbon/nitrogen balance and growth in both the organisms as these enzymes are known to generate glutamate needed for synthesis of several amino acids.

The organisms growing under aerobic condition frequently face oxidative stress due to interruption in normal metabolic processes. Thus cells under stressed condition are always threatened with the result of AOS formation. Considering the above fact, the level of oxidative stress in the fronds of *Azolla microphylla* and *Azolla pinnata* was analyzed following the exposure of plants to pretilachlor and enhanced levels of UV-B radiation applied separately and in combination. With the increasing doses of pretilachlor and UV-B the levels of superoxide radical (SOR) and hydrogen peroxide (H$_2$O$_2$) in both species were found to increased. Such pretilachlor treated fronds when subsequently exposed to UV-B$_1$ or UV-B$_2$ exhibited a continuous rise in the level of SOR and H$_2$O$_2$ with increasing UV-B dosage. The results also demonstrated that both the stress together caused more rapid generation of superoxide radical and hydrogen peroxide in both the species, but the contents were appreciably higher in *Azolla pinnata* than that of *Azolla microphylla*. 

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Accumulation of AOS under imposed oxidative stress is potentially a lethal situation, thus rapid reactions of AOS with lipids and proteins results in lipid peroxidation and damage to proteins, respectively which ultimately affect the membrane stability. Pretilachlor and enhanced UV-B radiation applied separately and in combination induced the MDA content in both species of Azolla fronds, thereby indicating enhanced lipid peroxidation. Both the stresses also showed that protein oxidation was stimulated by increasing doses of pretilachlor and enhanced UV-B radiation applied alone and in combination. It is also evident that pretilachlor and enhanced levels of UV-B radiation induced lipid peroxidation and carbonyl formation was greater in Azolla pinnata than in Azolla microphylla. The overall results demonstrate that greater accumulation of hydrogen peroxide and also to certain extent superoxide radical in the pretilachlor and UV-B treated fronds damaged cellular membranes appreciably which led to the reduced membrane stability index in fronds of Azolla pinnata than Azolla microphylla.

Pesticide and UV-B stress resulted in the formation of AOS in Azolla microphylla and Azolla pinnata and triggered an antioxidant system including enzymatic (SOD, CAT, POD, APX, GST, GR and DHAR) and non-enzymatic antioxidants (proline, ascorbate, non protein thiol, flavonoids and anthocyanin contents), to mitigate the toxic effect produced by free radicals. Azolla microphylla and Azolla pinnata exhibited an increase in SOD activity; which was more marked in Azolla microphylla. In Azolla pinnata, SOD activity increased in concentration dependent manner of pretilachlor, but decreased significantly at 10 and 20 µg ml\(^{-1}\) of pretilachlor, when combined with UV-B\(_1\) and at 5, 10 and 20 µg ml\(^{-1}\) pretilachlor together with UV-B\(_2\); however, the activity was still higher than that of untreated samples. Similar to SOD activity, CAT activity increased in both the species due to pretilachlor and UV-B stress but a declining trend was noticed at 10 µg ml\(^{-1}\) pretilachlor + UV-B\(_2\) and at 20 µg ml\(^{-1}\) pretilachlor + UV-B\(_1\) as well as 20 µg ml\(^{-1}\) + UV-B\(_2\) combination in Azolla microphylla. The stimulation in guaiacol peroxidase
(POD) activity in *Azolla microphylla* and *Azolla pinnata* fronds was also observed under tested stress. Among the other enzymatic antioxidants, ascorbate peroxidase activity in *Azolla microphylla* and *Azolla pinnata* fronds treated with pretilachlor and enhanced levels of UV-B radiation was also studied. An increment in APX activity of *Azolla microphylla* at 5 and 10 and 20 µg ml\(^{-1}\) of pretilachlor and enhanced levels of UV-B radiation (UV-B\(_1\) and UV-B\(_2\)) applied separately and in combination was recorded except 20 µg ml\(^{-1}\) pretilachlor together with UV-B\(_1\) or UV-B\(_2\) doses. In *Azolla pinnata*, APX activity increased only with 5 µg ml\(^{-1}\) pretilachlor, UV-B\(_1\) and UV-B\(_2\) doses applied separately, and in combination, however, it exhibited declining trend with 10 and 20 µg ml\(^{-1}\) pretilachlor alone, and in combination with UV-B\(_1\) as well as with UV-B\(_2\). The study also suggests that APX activity in *Azolla microphylla* fronds was considerably greater than that of *Azolla pinnata* fronds under all the tested doses of both the stresses. Almost similar trend in GR activity was shown by *Azolla microphylla* and *Azolla pinnata* fronds under similar conditions. In the fronds of *Azolla microphylla* and *Azolla pinnata* the GST activity enhanced progressively with rising doses of pretilachlor, alone and together with enhanced UV-B radiation. In contrast to GST activity, DHAR activity exhibited a declining trend in both species of *Azolla* under pesticide and UV-B stress.

In addition to enzymatic antioxidants, pretilachlor and enhanced UV-B radiation also raised the levels of non-antioxidants in *Azolla microphylla* and *Azolla pinnata* fronds. Both species showed significant enhancement in accumulation of proline, non protein thiol, UV-B absorbing pigments and anthocyanin contents under tested stress. However, the enhancement was greater in *Azolla microphylla* than that of *Azolla pinnata*. The continuous decline in ascorbate content was also observed in response to pretilachlor in both the species which further intensified in combination with enhanced levels of UV-B radiation. The overall study of enzymatic and non-enzymatic antioxidants demonstrates that the quantified antioxidants in *Azolla microphylla* fronds
were always greater than *Azolla pinnata* fronds under all the studied conditions.

In conclusion, pretilachlor a commonly used herbicide in rice field proved to be toxic for nitrogen fixing biofertilizer *Azolla microphylla* and *Azolla pinnata* at its recommended doses. Enhanced UV-B radiation and pretilachlor, when applied separately and in combination also adversely affect the growth and photosynthetic activity of *Azolla microphylla* and *Azolla pinnata*. The strong inhibitory effect on growth could be correlated with pesticide and UV-B induced inhibition of photosynthetic oxygen yield and photosynthetic electron transport activities. However, in *Azolla pinnata* photosynthesis showed greater sensitivity to pesticide and enhanced UV-B radiation than *Azolla microphylla*. PSII activity was greatly affected and PSI activity was least affected. Nitrogen metabolism of both *Azolla* species also affected due to these stresses and the effect was more prominent in *Azolla pinnata*. The study also concludes that pesticide and enhanced UV-B radiation, alone and in combination induce accumulation of AOS that caused considerable damage to lipids and proteins, the important constituents of biological membranes and thus caused substantial decrease in growth, photosynthetic activities and also activity of enzymes. Under pretilachlor and enhanced UV-B treatments, significant increase in SOD, CAT, POD, APX, GST, and GR activity as well as in proline, flavonoids and anthocyanin contents was noticed. The increase in these enzymes and metabolites which form strong antioxidant network may be related with tolerance of oxidative stress in both *Azolla* species. Further, under pretilachlor and enhanced UV-B treatments, decreased activity of DHAR and content of ascorbate showed that these are sensitive to these stresses which might have influenced proper scavenging of AOS as evidenced by data of lipid peroxidation, protein oxidation and membrane stability index.

The present study is thus of significant relevance as both *Azolla* species play an important role in the nitrogen economy of the paddy field and analysis of
the harmful effects of pretilachlor and enhanced UV-B alone and their interactive effects on physiological and biochemical metabolism of *Azolla microphylla* and *Azolla pinnata* is of great importance. In the last, we can also conclude that although both species of *Azolla* showed overall reduction in growth but *Azolla microphylla* is quite good in resisting against the stress caused by pretilachlor and enhanced levels of UV-B radiation and thus, can be used as biofertilizer even under such unavoidable stress condition.