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Genetic Resistant Cultivars Less Dependent on Agrochemicals ^(**)

1. INTRODUCTION

Agriculture in the advanced countries calls for management precision to sustain the high yield levels. Agriculture in the developing countries, above all, calls for management practices to increase productivity.

In short, world agriculture calls for increasing techno-scientific organization in order to: a) oppose and block the biotic and abiotic factors threatening the production; b) exploit the potential of the available cultivars; c) breed better cultivars by using genetic resources, and traditional and modern methodologies offered by the advancement of the sciences.

An important task of programmes in both developed and developing countries is concerned with shortening the dependence of crops on agrochemicals. Agrochemicals are essentially used to: a) protect crops from noxious insects and pests; b) control weeds; c) directly increase the productivity of crop plants.

A multidisciplinary approach, with genetics playing a key role, is necessary if an economical plant production is to be attained. Proper genetical manipulation of plants allows the incorporation of desired hereditary factors into a genotype, which will be endowed with a number of intrinsic characteristics that will make it less dependent on extrinsic inputs, such as synthesized chemical compounds, thus reducing expenses and possible harmful effects on the environment.

The present paper mainly deals with genetical procedures suitable to endow crop plant genomes with factors that will make them less dependent on agrochemicals; special attention will be given in this context to modern genetic methodologies.

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2. CROPS LESS DEPENDENT ON FUNGICIDES AND PESTICIDES.

Among the first successful applications of plant breeding techniques to obtain a food crop resistant to disease we might mention that set up in India in 1906 by a wheat breeder, Albert Howard.

2.1. *Genetic materials constituted by conventional plant breeding.*

At present a number of programmes on germplasm evaluation, intra and interspecific crosses and selection are carried out at the International Agricultural Research Centres on the major tropical food crops, and a long list of achievements could be produced. A few examples in different crops will elucidate the situation, although the list is far from being exhaustive.

— In *bean*: a number of lines (var. Rubona 5 in Ruanda and BAT 85 in Zambia) with good resistance to the Bean Common Virus (BCMV) have been recently isolated by CIAT scientists.

— In *cowpea*: a line (IT 82 D 716) characterized by resistance to or tolerance of eight diseases and two insects, in addition to good quality and yield, has been recently isolated at IITA.

— In *chick-pea*: ICARDA has selected ILC - 3279, resistant to *Ascochyta* blight (*Ascochyta rabiei*).

— In *broad bean*: ICARDA has a number of advanced lines resistant to chocolate spot (*Botrytis fabae*), *Ascochyta* blight (*Ascochyta fabae*) and rust (*Uromyces fabae*).

— In *potato*: CIP has developed a population that combines four sources of specific resistance to bacterial blight (*Pseudomonas solanacearum*).

— In *rice*: a multiple resistant line (IR - 36) has been isolated at IRRI. It shows resistance to various pests and diseases (blast, bacterial blight, tungro, gall midge, stem borer, brown plant hopper, green leaf hopper) and is now cultivated on 11 million hectares, where it has also proven to perform well in terms of yielding ability and early maturing. IR 42 and IR 50, two additional IRRI lines, show multiple resistance. Also IITA has obtained lines that resist the Rice Yellow Mottle Virus (RYMV).

— In *wheat*: many varieties of common and durum wheats that are highly productive, short-seasoned and, above all, resistant to rusts and mildew, have contributed to the successes of the first green revolution. CIMMYT has recently obtained varieties (Babwhite and Sunbird) that show good resistance to *Septoria tritici*, while a major research project regarding resistance to BYDV is now in progress.

— In *maize*: CIMMYT has developed a variety (Santa Rosa 8073) resistant to *Spiroplasma* spp (stunt disease) and another (Across 8072) resistant to *Perono-*

sclerospora. Again IITA is currently selecting several lines with resistance to Maize Shake Virus (MSV).

— In *barley*: ICARDA has selected three two row varieties (Faiz, Rhoi, and Taj), which are resistant to net blotch, stripe disease and powdery mildew, respectively.

— In *sorghum*: workers at ICRISAT selected from the germoplasm collection the variety Fromida, which is resistant to *Striga*; this variety is currently being grown in Burkina Faso.

— In *millet*: the variety WC 75 from ICRISAT is highly resistant to downy mildew.

In addition we mention some results recently obtained in Italy. New bush, dry bean varieties (Monviso, Morena, Montecarlo, Monterosa) resistant to both Bean Common Mosaic Virus (BCMV) and Halo blight (*Pseudomonas phaseolicola*) have been selected in Italy at the Experiment Institute for Vegetable Crops, at Montanaso Lombardo, and released to the farmers.

2.2. *Mutagenesis.*

Mutation breeding may induce desirable genes that confer high-type resistance or tolerance, and may break undesirable linkages involving genes for disease resistance. Moreover, the prospect of changing a few characters in an otherwise unchanged genetic background, as might be done by somatic mutation (somaclonal variation), is attractive especially in vegetatively propagated plants.

Among the numerous lines and varieties selected for improved disease resistance, with reference only to those released after 1980 one could mention: in bread wheat, 5 varieties from China and 1 from USA showing resistance or tolerance to stripe rust or *Gibberella*; in durum wheat, 1 variety from Austria having a short straw mutant Italian variety as a parent; in rice, 5 varieties, all from Eastern countries, generally characterized as resistant to *Pyricularia Oryzae*; in maize, three varieties selected in China as resistant to leaf spot; in barley: three varieties from Sweden, India and Denmark; in leguminous plants (bean, soyabean) 5 varieties resistant to rust or *Sclerospora* or Bean Golden Mosaic Virus.

More details and information can be found in *ad hoc* IAEA Publications (IAEA, 1971; Micke A. *et al.*, 1985).

2.3. *Advanced methodologies.*

An important new step, which opens entirely new perspectives, is represented by the recent use of advanced biotechnologies — at both cellular and molecular level — for the transfer and expression of resistance genes into new genotypes. Unfortunately, studies and knowledge regarding agricultural plants and their

parasites are still at a rather low level, so that the methodologies of genetic engineering cannot yet be applied on any extensive scale and one cannot expect any short-term results from them.

2.3.1. Recombinant DNA techniques.

As regards the recombinant DNA method, i.e. the introduction and stable integration of foreign genes into plants through engineered vectors, current research work is mainly concerned with combinations: cereals-rusts, potato-*Phytophthora infestans*, and flax-*Melampsora lini*, etc.

More extensive use of recombinant DNA technologies calls, above all, for a great deal of additional information on the character control in host plants (i.e., about the location and structure of specific genes in both resistant and susceptible species and varieties) and pathogens.

Since the genetic analysis of crop plants is made particularly difficult by the complexity of their genetic systems, it is likely that the first results will be obtained in characters controlled by single genes.

Genetic analysis of pathogenous fungi, on the other hand, is made difficult by the predominance of asexual reproduction. However it is encouraging to note that, thanks to the small size of their genomes and the ease with which they can be genetically manipulated, promising results have been obtained in a number of phytopathogenous bacteria. It has been possible to identify the molecular structure of and to clone the genes involved in the pathogenesis process. Particular mention should here be made of the work already done (and still continuing) on *Agrobacterium tumefaciens*, *Pseudomonas savastanoi*, *Pseudomonas solanacearum* and *P. syringae* var. *glycinea* (Staskawicz, 1983).

Cloning, together with the preparation of genome libraries, is making possible the physical isolation of the specific genes that — in both host and parasite — codify for gene products involved in the host/parasite relationships; this, in turn, ensures that these products can be identified and examined in detail, thus permitting a detailed analysis of the gene action.

Identification, analysis and isolation of the plant genes, together with detailed knowledge on their role, constitute a preliminary step for their transfer and expression into the genome of plants different from the ones in which they were first identified and isolated (including plants which are evolutionary divergent from them and cannot be hybridized with them). As is well known, the possible vectors for such transfers include plasmids of pathogenous bacteria (i.e., the Ti plasmid of *Agrobacterium tumefaciens*) and viruses, since they draw advantage from the natural phenomenon of the intimate association that exists between the microorganism (be it useful or pathogenous) and the plant.

2.3.2. « In vitro » culture of plant cells.

Another strategy of selection for disease is based on the hazard of a more casual type of variation coming from *in vitro* plant culture (meristems, anthers, embryos, calluses, cells, and protoplasts).

The useful genetic characteristics expressed in isolated cells and protoplasts (and to be found in plants regenerated from the cultivated cells, protoplasts and grain pollen) already include some examples of resistance to diseases. A cultivar of sugar cane resistant to the Fiji virus has been obtained by means of the selection of plants derived from multicellular calli; another plant was found to be resistant to both the Fiji virus and peronospora (*Sclerospora sacchari*). Tomato plants resistant to Fusarium wilt (*Fusarium oxysporum* f. sp. *Lycopersici*, race 2) were recently recovered from cell cultures using *in vitro* selection against phyto-toxin. These disease-resistant mutants proved to be inherited as single dominant gene.

Many plants with different degrees of resistance to *Phytophthora infestans*, including some with very high degree of resistance, have been regenerated from potato mesophyll protoplast, together with other variants in a number of agronomic characteristics. As is known, this genetic variability or somaclonal variation is due to genome modifications spontaneously occurring in *in vitro* cell culture and in regenerated plants and their progeny: aneuploids, polyploids, chromosome losses, chromosome breakage and/or rearrangement. These events can be enhanced and accentuated by chemical and physical mutagenic treatments.

Experimental mutagenesis combined with *in vitro* culture offers certain advantages. For example, dominant mutations are a relatively rare event, but *in vitro* mutagenesis in large populations will give an increased probability of obtaining such mutants. This would be particularly relevant for improving the disease resistance of cross-pollinated crop plants.

Another important method of transferring resistance genes, though once again haphazard in nature, is represented by protoplast fusion (Shepard, 1981), including intergeneric fusion. This method tends to become more positive as the number of translocations of genome segments (chromosomes or chromosome fragments carrying resistance) becomes greater. Mutagenic treatments can again prove effective in these cases. The method also involves the transfer of cytoplasm organelles, chloroplast and mitochondrial DNA, though the effects may vary and have to be evaluated case by case.

The potential of *in vitro* selection in connection with various pathogen types and to various host plant materials clearly warrants further attention to this method. It is one of the main attractions of *in vitro* techniques that they can provide a scale for selection that would be difficult, if not impossible, to achieve in other ways. A potato breeder, for example, could screen — in greenhouse or field — some 50,000 to 100,000 seedlings *per annum* for disease resistance. In the laboratory, on the other hand, 20 million protoplasts obtained from only 1 gram of leaf tissue, could easily be cultured. Supposing a given beneficial mutation to have a probability of 10^{-5} , one may expect one such mutant to occur among the 100,000 seedlings, while as many as 200 mutant plants are likely to be obtained from protoplast culture screening. However, a limitation derives from the fact that resistance may be needed at particular stages in the host's life cycle, so that

it does not matter whether or not it is expressed at other stages, at the cellular level for example. Another major handicap is that regeneration from *in vitro* cultures still poses problems for many important plant species.

Plants capable of vegetative propagation should derive particular benefit from the *in vitro* culture methods: they are not only subject to a large number of diseases, but also consist of genetically uniform clones (often fulfilling particular high consumer requirements) that represent a serious risk of vulnerability in the event of an epidemic. Beneficial genetic changes, by either spontaneous or induced mutations, that appear in *in vitro* culture, will be easily transferred in the regenerated plant and then transmitted by normal propagation methods.

2.3.3. Direct DNA transfer into plant cells.

The microinjection of genomic DNA fragments obtained from resistant forms into the cells or protoplasts of susceptible varieties would also seem to hold promise.

2.3.4. Increased pathogenicity of endopathogens.

Thanks to genetics, moreover, there is also another way of promoting lesser use of fungicides and pesticides, namely by increasing the pathogenicity of the insects, fungi, microbes and virus that parasite the noxious insects and pests.

Genetic improvement of the natural enemies of arthropods could be achieved by employing artificial selection, hybridization and genetic engineering. Selection for insecticide resistance in predatory insects, and the gene transfer techniques developed for *Drosophila*, may be applicable to beneficial insects, thus enhancing their role of biological control in integrated agricultural pest management systems (Hoy, 1985). Microbial and bacterial pesticides can also be obtained, by means of genetic engineering techniques. It would seem that such pesticides, given their lack of pathogenicity, their low capacity for genetic exchange and limited environment persistence, can be safely released into the environment.

Gene analyses, cloning and gene manipulation have already been performed on *Bacillus thuringiensis*, which forms a protein that is toxic for insects and at present is the best representative of the category of the entomopathogens.

The gene coding for the active endotoxin of *B. thuringiensis* Kurstaki was cloned into isolates of corn root colonising *Pseudomonas fluorescens* (Kaufman, 1986). The system *Agrobacterium radiobacter* - *Agrobacterium tumefaciens* is also well known: plant infection by the latter is prevented by the former (Napoli and Staskawicz, 1985). Several *P. fluorescens* isolates secrete antibiotics and protect plants from infections by root pathogens. *P. fluorescens* strain HV37a can control the *Pythium ultimum* induced damping-off disease of cotton (Guttererson and Warren, 1985). This *Pseudomonas* produces an antibiotic responsible for a significant proportion of disease control. Monsanto Co. is testing *P. fluorescens* as protectant of corn plants from the black cut worm, which affects the roots of

corn and soybeans. This *Pseudomonas* contains the gene from *B. thuringiensis* which control a toxin that is poisonous to insects (Sterling, 1985).

Biodegradation and detoxification of pesticides, herbicides and other environmental pollutants or toxic xenobiotic compounds can be obtained by using micro-organism and microbial systems isolated from soil and water. Genetic engineering of the genes controlling the enzymes produced by such microorganisms, for example, can increase the degradative capability of those bacteria.

However, other speakers at this meeting will undoubtedly have more to say about these biotechnological applications and I shall not therefore pursue the topic further.

3. CROPS TOLERANT TO HERBICIDES.

3.1. Glyphosate resistant genes.

Even though some very advanced genetical methodologies may be involved, the agrotechnical importance and the economic impact of varieties tolerant to herbicides undoubtedly take second place when compared to the aspect discussed in the previous section.

Quite apart from the well known advantages associated with the use of herbicides (less competition for fertilizers and water, reduction or elimination of soil tilling, and reduced energy consumption and production costs), the use of varieties tolerant to herbicides or the introduction of multiple resistance into crop plants could well make possible the use of new and more efficient herbicides for complete weed control, probably reducing the overall use of agrochemicals with consequent benefit for the ecological system as a whole.

The project in a few years' time (1990?) will very probably permit the registration of new varieties tolerant to herbicides is the one concerned with the glyphosate resistance ascertained in *Salmonella typhimurium* and transferable into dicotyledon plants. Glyphosate, which is extensively used in herbicides, blocks the synthesis of the aromatic aminoacids. Going into greater details, it kills plants by suppressing the activity of an enzyme, EPSP-synthase, which is needed for making essential aromatic aminoacids (phenylalanine, tyrosine, tryptophane). Researchers of Calgene Inc. (Comai *et al.*, 1983 and 1985), working with a mutant strain of *Salmonella typhimurium*, have isolated the gene *aroA* responsible for coding a protein different in a single aminoacid from the normal EPSP-synthase (5-enolpyruvylshikimate-3-phosphate synthase). This small change is already sufficient to make it less susceptible to inhibition by the herbicides. Using DNA-recombinant technique and *Agrobacterium tumefaciens* vectors, this nuclear gene has been transferred into tobacco cells from which entire plants have been recovered. In these transformed plants the *Salmonella* mutant gene was able to express and form the modified enzyme, so that the plants are tolerant to glyphosate.

However, these plants are not completely resistant, probably because the bacterial enzyme product ends up in the cell cytoplasm, because its gene lacks the

sequence for transport to the chloroplasts, where the other enzymes of the synthetic pathway are located. It has been found that the enzyme produced by the *aroA* gene represents 25% of the total EPSP synthase activity measured in the leaf extracts of the transformed plants.

Experiments for improving the *aroA* constructs and transferring this *aroA* nuclear gene also into other crops, (soybean, cotton, tomato, seed oil rape, and poplar trees) are now in progress at Calgene. Significant efforts are also being made to use the *aroA* gene to transform corn plants.

Working with petunia, researchers from Monsanto Co. have hooked the EPSP synthase gene to a viral regulation sequence that is a very active promoter of gene expression, and they have thus succeeded in culturing petunia cells that resist glyphosate on account of the larger transcriptional activity of the EPSP-synthase gene that causes an overproduction of the enzyme. Whole plants are far more resistant to the glyphosate than the control plants, this being due to the fact that the hyperactive gene preserves the chloroplast transport sequence.

One can therefore visualize a degree of resistance completely suitable for commercial varieties being obtained by combining the two genetic engineering methods just outlined, i.e., using the glyphosate-resistant *Salmonella* mutant and making it hyperactive.

3.2. Other herbicide resistant genes.

There are also indications that genetic engineering can be used for making crops resistant to other herbicides.

Atrazine, for example, is a member of the triazine group, which kills plants by blocking the action of the chloroplast proteins needed for photosynthesis (photosystem II) and coded by the chloroplast gene *psb-A*. Atrazine is the main herbicide used for spraying corn fields, but could be toxic for dicotyledon plants (soybean, for example) grown in rotation with corn, since the compound tends to persist in the soil and also contaminates the ground water.

Corn and pigweed are two out of several plants that are naturally resistant to atrazine. This characteristic is controlled by the chloroplast gene *psb-A* that codes for a 34-KD chloroplast membrane protein which is not inhibited by atrazine, by changing a single aminoacid in the protein sequence.

The protein involved in the electron transfer at the reducing site of photosystem II of *Clamydomonas reinhardtii* has altered the aminoacid sequence at the herbicide binding site in comparison to the analogous protein in the atrazine susceptible plants. (Erickson *et al.*, 1985).

Newly introduced genes are usually incorporated in the nuclear genome and chloroplast genes do not have the correct control sequences to be expressed there. Nevertheless, Bogorad's group, in collaboration with that of J. Schell at the Max Planck Institute for Plant Breeding Research in Cologne and M. van Montagu at the State University of Ghent in Belgium, has now introduced the resistant form of the gene into tobacco plants, which thus become capable of withstanding atrazine treatment, though Bogorad notes that the plants did not appear to be completely

healthy. The investigators first attached the gene to a regulatory sequence, which enables it to be expressed in the nuclear genome, and also to a chloroplast transport sequence.

The transfer of isolated chloroplast *psbA* gene that confers resistance to photosystem II directed herbicides (e.g., diuron and atrazine) from algae (e.g., *Chlamydomonas reinhardtii*) to other plants has been proposed (Mets, 1985).

One could envisage and explore a practical way of transferring herbicide resistance through chloroplasts transferred by means of protoplast fusion. The transfer of chloroplast genes between sexually incompatible plants could be obtained by means of genetic recombination between chloroplast genomes.

3.3. Selection of naturally occurring mutations for herbicide resistance.

Another advanced biotechnology, plant cell *in vitro* culture, can be used to screen for natural spontaneous herbicides resistance. Mutant selection in populations of protoplast-derived colonies has brought to light some triazine-resistant *Nicotianae*, which have been developed into whole plants (Maliga, 1985).

R. Chaleff and T.B. Ray of Du Pont (quoted by Moffat, 1985) have isolated several tobacco mutant cells capable of resisting sulfonylurea. This resistance appears to be due to an altered acetolactate-synthase, which (according to Falco of Du Pont at the Conference on Plant Biotechnology held at Cornell University in 1985) is expressed also in the whole plants. Somerville *et al.* (1985) isolated several mutants of tobacco resistant to the herbicides chlorsulfuron and sulfometuron by direct selection in tissue culture. The basis of resistance appears to be an alteration in the properties of acetolactate-synthase which renders the enzyme relatively insensitive to inhibition by the herbicides.

Sulphonylureas exert the same effect on the branched chain of aminoacids as another class of herbicides, the so-called imidazolinones. A maize cell line resistant to this group of herbicides has been found by Shaner of American Cyanamide, Princeton, N.J., who was able to regenerate whole plants from this mutant (Applied Genetics News, 6/2 pag. 8, 1985).

4. CROPS LESS DEPENDENT ON FERTILIZERS.

Maintenance or enhancement of yield, concomitant with a reduction of external inputs, calls for a positive increase in the internal factors governing productivity. This result could be obtained either by improving the genetic basis of the numerous physiological traits connected with yield potential (from photosynthesis and ion uptake to biosynthesis, from transport to accumulation of photosynthates), or by improving the efficiency of fertilizer application and uptake.

Since the second of these approaches is closely related to problems and progress in agricultural chemistry and agronomy, I shall here limit myself to a few remarks regarding the first of the two approaches.

Basic studies indeed are in progress. For instance: the possibility of improving photosynthesis by increasing the carbon dioxide fixing ability is studied, but still at a plant cell level (Anonymous, 1986); genetic analyses of nitrate reductase activity in wheat and *Sorghum* are advancing (Gallagher *et al.*, 1980; Mishra *et al.*, 1981); the DNA sequence of an A gliadin gene isolated from a genomic clone library has been determined (Greene *et al.*, 1985), and so on. Firstly, since genetic knowledge of the complex processes involved in these plant activities is as yet wholly inadequate, the application of advanced genetic technologies will be delayed. On the other hand, scientists are trying to approach the final aim throughout multidisciplinary investigation of the other side of the dilemma: the fertility elements and the related problems. Thus, great and increasing attention is being paid to the nitrogen supply and its biological fixation and the genetic control of this function, trying to reduce the dependence for plant growth and productivity from this essential element, which is undoubtedly the major limiting factor in crop yield.

4.1. Biological Nitrogen Fixation.

Some plants, notably grain crops, rely mostly on combined N_2 sources, some other plants, notably legumes, are at least partially self-sufficient through symbiotic N_2 -fixation.

The ability to fix gaseous nitrogen is restricted to certain prokaryotes (cyanobacteria, actinomycetes, bacteria) that contain the nitrogen fixing enzyme nitrogenase. While a number of these organisms fix atmospheric nitrogen in the free living state, some form symbiotic associations with higher plants, like: legume-*Rhizobium*, nonlegume (*Alnus*, *Casuarina*)-actinorhiza (Frankia), and non-legume (Azolla)-cyanobacteria (*Anabaena azollae*).

Incidentally, among the leguminous plants, the nodulating trees and shrubs, like *Acacia*, *Sesbania*, and *Leucaena*, may have potential for expanded use, especially in the III world if, as said, "the green revolution in Africa will be based on seeds and trees".

As is well known, the genetic system in the nodulant microorganisms is controlled by the genes *nif* (nitrogen fixation), *hup* (hydrogenase uptake), *nod* (nodulating) (Haaker and Verger, 1984; Dilworth and Glenn, 1984). On the other side, the genetic system which in leguminous plants allows symbiosis and nodulation, involves the *leg Hb* genes (which synthesize the globin fraction of the leghemoglobin protein) and the "nodulin" genes. Of course, classical selection and breeding techniques (as well as agronomical and microbiological procedures to overcome limiting factors like soil acidity, drought, low temperature, low phosphorus and high iron in water and soil nutrient level, and to improve technologies for production and use of inocula) can be applied very effectively to the leguminous-nitrogen-fixing bacteria associations.

Interesting data and suggestions come from some of the most recent researches. Genotypic differences have been found in a increasing number of species. A *Rhizobium* strain (RRIM 56) interacts differently with winged-bean

(*Psoralea tetragoloba*) genotypes: symbiotic effectiveness in some lines is double that in others; N_2 fixation varies from 326.8 up to 2,066.8 μ mol per plant per hour. Studies on inheritance and heritability of nodulation and N fixation confirm the possibilities of very positive improvement for these characters in winged-beans (Iruthayathas *et al.*, 1985).

Some soybean varieties showed more effective symbiosis with some mutant strains of *Rhizobium* than other ones (Sapra *et al.*, 1984; Baendia-Claveria and Luiz-Sainz, 1985). Some strains of *Rhizobium* inoculated on soybean increased seed yield by 10%, comparable to an application of 30 Kg. N_2 /ha (Kao, 1983). An EMS-mutant of soybean (nts 382) showed supernodulation in mass and number, several times higher than in the mother type (Carral *et al.*, 1985). The variety Cowpea-85 is superior to a set of other varieties for its response to *Rhizobium* inoculation, whilst the *Rhizobium* strain C 41 significantly increases number and weight of nodules, plant height, yield of fodder crop and dry matter in cowpea (Soboo *et al.*, 1984). Quite recently, genes controlling nodulation and function have been described for the first time also in chickpea (Davis *et al.*, 1986). Research on *Rhizobium* symbiosis in mungbean has confirmed the great variability in nodulating capacity; strain BM-301 resulted in the highest number of nodules/plant (19), highest nodule/dry weight (13mg/plant) and the highest seed yield (1139 Kg/ha) (Shahjahan and Islam, 1984). Similarly, groundnut varieties have been selected showing better nitrogenase activity and nodule mass (Arunachalam *et al.*, 1984). And the list can go on with interesting suggestions and perspectives on the genetic improvement of both partners, plant and bacterium, for selecting new varieties less dependent from nitrogen supply.

Worthy of being developed should be also studies involving the use of ^{15}N as a tracer, and of the traits in legumes that allow them to use simultaneously fixed N_2 and soil N_2 . On the other hand research on fast growing nitrogen-fixing trees and shrubs, and on associated bacteria, should be pushed on especially for the developing countries of the arid regions of the planet, in which wood and food are of primary importance. All these results reveal the importance of plant genotype and suggest the possibility of improvement of N_2 -fixing symbiotic associations through breeding new and more suitable nodulating species and varieties by plant breeding.

Moreover, breeding techniques might also be used for obtaining varieties of cereals and other graminaceae (non-nodulating species), and perhaps monocotyledons in general, suitable for a more efficient rhizosphere or as mycorrhizal associations, which may enhance the activity of the N_2 fixing bacteria and the general amount of N_2 made available for non-nodulating crops.

In the roots of forage grasses, of sugar cane, of cereals (e.g., rice, wheat) bacterial associations and consequent nitrogen fixation are known (Neyra and Döbereiner, 1977), although the exact nature of the grass-bacteria association is still unclear.

Waiting for the possibilities of developing N-self-sufficiency in graminaceous species, maximization of N_2 fixation through plant bacteria association, studies

of plants (trees included) and associated bacteria for their ability to promote N_2 -fixation and accumulation of dry-matter, identification of the various limiting factors, and elaboration of agronomic practices to enhance or promote N_2 -fixation, will certainly be, especially for the Third World agriculture, another approach towards less dependence on N_2 -fertilizers. Of course many of these problems are not inherent to my report, and certainly the great role of the rhizosphere and the mycorrhizae will be examined by other authors.

Nevertheless plant genetics and breeding can be considered as a promising tool for achieving better non-leguminous non-nodulating plant-bacteria association if we take into account some preliminary data.

Genotypic differences in nitrogenase activities have been observed in *Paspalum notatum*, *Pennisetum purpureum* and wheat (Döbereiner and Day, 1975; Larson and Neal, 1976). In maize some S lines were 10 to 20 times higher in nitrogenase activity than the original cultivar and significant heterosis effects were ascertained in crosses between higher-fixing versus less-fixing cultivars (von Bölow et al., 1976). A disomic substitution line of wheat, grown for 30 years without nitrogen supply, showed in its rhizosphere a highly specific association with a *Bacillus* sp.; on the other hand the rhizosphere contained more nitrate-reducing bacteria and a lower total number of microorganisms (Larson and Neal, 1976). Of course, the important way of reducing nitrogen fertilizers through symbiosis like the *Azolla-Anabaena azollae* association, so useful for rice growing, or other sources of N_2 -fixation through the activity of free-living microorganisms (e.g., *Cyanoobacteria*), cannot be discussed in this report because no genetic improvement of the crop varieties directly acts on a major availability of organic nitrogen.

4.2. Advanced methodologies for enhancing biological nitrogen fixation.

Studies on location, structure, regulation product and function of the *nif* and *nod* genes are in progress. Molecular genetic analysis of *nif* genes of *Klebsiella pneumoniae*, of *Rhizobium* and other bacteria is progressing but several important points need to be understood.

The different aspects of the regulation of symbiotic and non-symbiotic *nif* genes might enlighten the possibilities of improving the efficiency of nitrogen fixation in various crops. Still unknown is the mechanism of action of the products of the *nod* genes: the DNA region encoding nodulation and host range functions in some *Rhizobium* species is small, whilst in others (*R. meliloti*, for instance) several DNA regions appear involved.

Genetic engineering is already being applied to search for *Rhizobium* strains more efficient in N_2 -fixation. Symbiotic properties can be transferred on plasmids among *Rhizobium* and other nitrogen fixing bacteria. Expression takes place, although the nodulating capacities and the degree of N_2 -fixation is still under study. Examples of *nif* and *nod* genes transferred from nitrogen fixing bacteria to non-nitrogen fixing prokaryotes and eucaryotes are already available. As a matter of fact, transfer and functioning of the *nif* genes to closely related organ-

isms (e.g., the prokaryote *E. coli*) is rather easy and known since 1972 (Dixon and Postgate, 1972). On the contrary, limited expression or expression subjected to other conditions (e.g.: absence of O_2) was ascertained in case of transfer to other non-nitrogen-fixing microorganisms (e.g., the eukaryotes yeasts).

In this situation, the possibility of obtaining in non nodulating crop plants, varieties able to fix N_2 via *nif* genes transferred in cells, does not seem achievable in an immediate future. Such change will imply a profound modification of its biochemical and biophysical architecture if we only take into account the tremendous energy requirement for N_2 -fixation. It will interfere with other energy demanding processes, sacrificing also productivity (20% yield reduction has been calculated), unless plants with greater photosynthetic capacities might be genetically built.

CONCLUSION

Synthesis of new cultivars less dependent on agrochemicals is probably the major task in the programs for introducing in the genome of crop plants resistance genes to biotic and abiotic stresses.

Plant breeders have to work very hard; fortunately they are supported by the progress of fundamental studies and by the elaboration of new, often interdisciplinary, methodologies.

The most advanced genetic technologies are also progressively applied and, in due time, relevant results will appear.

The ample cooperation among geneticists, breeders, agronomists, physiologists and entomologists, microbiologists, biochemists, enzymologists and biophysicists is a guarantee that renewable resources produced by agriculture will be increasingly grown and obtained with constantly decreasing utilisation of non renewable resources.

Availability of new and better plants, more economical, more suitable to agro-food and agro-energy filieres, more in harmony with the ecosystems, is a real contribution of genetics and breeding for the agriculture of the Third World and of the entire planet.

REFERENCES

- Anonymous (1986) - Increasing carbon dioxide fixing ability of plant cell culture by introducing ribosomes into protoplast and regenerating. *Advan. Develop. Res. Inst. In: «Derwent Biotechnology Abstracts» Abstr. 86-04913.*
- ARUNACHALAM V., PUNGLE G.D., DUTTA M., NAMBIAR P.T.C. and DARY P.J. (1984) - Efficiency of nitrogenase activity and nodule mass in predicting the relative performance of genotypes assessed by a number of characters in groundnut (*Arachis hypogea*). «*Experimental Agriculture*», 20 (4), 303-309.
- BURDIA-CLAVIERIA A.M. and RUIZ-SAINZ J.E. (1985) - Isolation of mutants of fast-growing soybean strains that are effective on commercial soybean cultivars. «*Physiologia Plantarum*», 64 (4), 507-512.
- CARRAL B.J., McNEIL D.I. and GRESSHOFF P.M. (1985) - Isolation and properties of soybean (*Glycine max* (L.) Merr.) mutants that nodulate in the presence of high nitrate concentrations. «*Proc. Nat. Acad. Sci. USA*», 82 (12), 4162-4166.
- COMAI L., SEN L.C. and STALKER D.M. (1983) - An altered *aroA* gene product confers resistance to the herbicide glyphosate. «*Science*», 221, 370-371.
- COMAI L., FACCIOOTTI D., HASTY W.R., THOMPSON G., ROSE R.E. and STALKER D.M. (1985) - Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. «*Nature*», 317, 741-744.
- DAVIS T.M., FOSTER K.W. and PHILLIPS D.A. (1986) - Inheritance and expression of three genes controlling root nodule formation in chickpea. «*Crop Science*», 26 (4), 719-723.
- DELWORTH M. and GLENN A. (1984) - How does a legume nodule work? «*TIBS*», Dec. 1984, 519-521.
- DORREINER J. and DAY J.M. (1975) - In: *Nitrogen fixation by free-living microorganisms* (W.D.P. Stewart, ed.), pp. 39-56. Cambridge Univ. Press, London.
- DEON R.A. and POSTGATE J.R. (1972) - Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. «*Nature (London)*», 237, 102-103.
- ERICKSON J.M., RAHIRE M., ROCHAUX J.D. and MEYS L. (1985) - Herbicide resistance and cross-resistance: changes at three distinct sites in the herbicide-binding protein. «*Science*», 228, 204-207.
- GALLAGHER L.W., SALEMAN K.M., QUALART C.O., HUFFAKER R.C. and RAINS D.W. (1980) - Major gene control of nitrate reductase activity in common wheat. «*Crop Science*», 20, 717-721.
- GREEN F.C., ANDERSON O.D., LITTS J.C., GAUTHER M.F. (1985) - Control of wheat protein biosynthesis. «*Cereal Chem.*», 62 (5), 398-405.
- GUTHRIESON N.I. and WARREN G.J. (1985) - Regulation of antibiotic biosynthesis in *Pseudomonas fluorescens* strain HV32a. «*Phytopathology*», 75 (11), 1325.
- HAAXER H. and VEDGER C. (1974) - Enzymology of nitrogen fixation. «*TIBS*», April 1984, 188-191.
- HOY M.A. (1985) - Developing insecticide resistances in insect and mite predators and opportunities for gene transfer. «*Abstr. Pap. Am. Chem. Soc.*», 190 Meet., AGRO29.
- IAEA (1971) - *Mutation breeding for disease resistance*, Vienna, Austria.
- LEUTHYATHAS E.E., VLASSAK K. and LAEREMANS R. (1985) - Inheritance of nodulation and N₂ fixation in winged bean. «*Journal of Heredity*», 76, 237-242.

- KAO T.C. (1983) - *Studies on the biological nitrogen fixation of soybean nodules* «Memoirs of the College of Agriculture, Nat. Taiwan Univ.», 23 (1), 96-99.
- KAUFMAN R.J. (1986) - *The development and risk assessment of a genetically engineered microbial insecticide*. «J. Cell Biochem.», Suppl. 10C, 94.
- LARSON R.I. and NEAL Jr. J.L. (1976) - *Environmental role of nitrogen-fixing blue-green algae and symbiotic bacteria*. Int. Symp., Uppsala.
- MALIGA P. (1985) - *Cell culture approaches to obtain herbicide resistant chloroplasts in crop plants*. «Abstr. Pap. Am. Chem. Soc.», 190 Meet., AGRO 27.
- MEYS L.J. (1985) - *Single celled photosynthetic organisms as a source of herbicide resistance genes*. «Abstr. Pap. Am. Chem. Soc.», 190 Meet., AGRO 24.
- MICKI A., MALUSZYNSKI M. and DORNI B. (1983) - *Plant cultivars derived from mutation induction or the use of induced mutants in cross breeding*. Mutation Breeding Review, n. 3.
- MISHRA S.P., SINHA S.K. and RAO N.G.P. (1981) - *Genetic analysis of nitrate reductase activity in relation to yield heterosis in sorghum*. «Zeit. für Pflanzen.», 86, 11-19.
- MOFFAT A.S. (1985) - *Herbicide resistant crops to be first recombinant plant products*. Genetic Engineering News, October 1985, p. 16.
- NAPOLI C. and STASKAWICZ B. (1985) - *Molecular genetics of biological control agents of plant pathogens: status and prospects*. «Biol. Contr. Agric. IPM. Syst. Meet.», 455-463.
- NEYRA C.A. and DÖRRREINER J. (1977) - *Nitrogen fixation in grasses*. «Advances in Agronomy», 29, 1-38.
- SAPRA V.T., FLOYD M., GARNER R. and MOORMERJI S. (1984) - *Evaluation of soybean germ-plasm for stress tolerance and biological efficiency*. «Nitrogen fixation. Soybean Genetics Newsletter», 11, 76.
- SHAHJAHAN Md. and ISLAM R. (1984) - *Preliminary screening of Rhizobium strains for mung-bean*. «Bangladesh Journal of Agricultural Research», 9 (2), 130-135.
- SHEPARD J.F. (1981) - *Protoplasts as sources of disease resistance in plants*. «Am. Rev. Phytopath.», 19, 145-166.
- SOROKO M.S., CHAHAL V.P.S. and BEER S.M. (1984) - *Effect of Rhizobium inoculation on different varieties of cowpea (Vigna unguiculata L. Walp.)*. «Journal of Research, Punjab Agricultural University», 21 (2), 316-319.
- SOMERVILLE C.R., HAUGHN G. and MAZUR B. (1985) - *Selection for herbicide resistance at the whole plant level*. «Abstr. Pap. Am. Chem. Soc.», 190 Meet., AGRO 25.
- STASKAWICZ B.J. (1983) - *Molecular genetics of plant disease*. In: *Biochemical plant pathology* (Ed. Callow J.A.), John Wiley and Sons, Chichester, pp. 199-213.
- STOKLINO J. (1985) - *Montanto to perform first bio-pesticide field test*. «Genetic Engineering News», 5 (2), 1.
- VON BÜLOW J.F.W., DÖRRREINER J. and PODESTÀ J.A. (1976) - *11a Reunio Brasileira Milho Sorgo, Piracicaba, São Paulo*.