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DISCUSSION ON THE POSSIBILITIES OF PRODUCING MUTANTS RESISTANT TO PERONOSPORA TABACINA (BLUE MOLD DISEASE) OF TOBACCO PLANTS BY SEED TREATMENT WITH IONIZING RADIATION

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Various investigations were performed against the blue mold disease or peronospora tabacina which causes great damages on tobacco plants. Three types of approaches to solve this problem are seen in literature: I) Developing methods to destroy the mold (Peronospora tabacina), II) Investigation on the favorable conditions for the germination of peronospora tabacina and its lethal action, III) Producing disease resistant tobacco plants by a) vaccination, b) hybridrization, c) producing mutants with chemicals and radiation.

This present report is a survey from literature which will deal mainly on the possibilities of obtaining disease resistant mutants through the use of ionizing radiation, after describing briefly the other methods.

I) *Developing methods to destroy the mold (Peronospora tabacina)*

The first approach on which most investigations were made is the use of fungicides. Streptomycin sulfate is among the most widely used antibiotic,[1] while, according to some literature, Maneb (Mangan etilen dithiocarbamat) is preferred among the chemicals used [2,3]. Lately, a relationship between chemical constitution and fungical properties was worked out. Aromatic compounds containing halogen substituents activated by the presence of electronegative group in ortho and para (mainly nitro group), are found to be fungitoxic. The labile halogen is considered to react with an essential amino acid or mercaptan group of the microorganism.[4] Further work using dessicating agents causing the drying of the intra tissue growing mycelium of Peronospora tabacina are seen.[5] It was also reported that treatment of tobacco leaves by Co 60 gamma rays prevented the growth on the mold of tobacco.[6]

Although some results were obtained, these different approaches to destroy the pest did not solve the problem of the blue mold disease.

II) *Investigation on the favorable conditions for the germination of Peronospora tabacina and on its lethal action*

Meteorological factors have a primary role on the propagation of the disease. Sporulation occurs in the early hour of the morning. Incubation period required for sporulation is divided into relative long one of vegetative growth and conidiophores and short period of conidia formation and maturation. The conidiophore is dependent of optimal humidity (97%) in its early steps of development. A 24 hour favorable for sporulation will be in which day temperature do not exceed 30^{o}C for more than 6 hours and humidity on the following night exceed 95% for a minimum continuous period of 3 hours.[7]

It was also observed that riboflavin played an important role on the germination of Conidia of Peronospora tabacina on leaves of Nicotiana tabacum. 1.7% of germination was reported to increase to 44% if riboflavin was added to the leaves. Washing the leaves prior applying riboflavin increased the germination to 95%. So water soluble compounds inhibitory to germination were extracted from tobacco leaves. Their analysis showed that they were phenolic in nature.[8] Also the chemotactic action on the inhibition of germination of tobacco plants of Peronospora tabacina was investigated by paper chromatography of its spore extracts.[9]

III) *Producing disease resistant plants*

a) Vaccination

A relationship between the nutritive condition and the resistance to the disease was investigated. It was observed that the increase of Nitrogen in soil was favorable for acquiring resistance to the mold.[10] On the other hand, it was observed by Angel (1957) and Port (1959) that tobacco plants having slight infections on their stems do not develop foliage disease contrary to their neighboring plants having no stem infection. This fact gave Mandryk the idea of obtaining disease resistant plants by vaccination, and experiments were carried out in the following way: Tobacco seedlings were subjected to benzol vapour each night for two or three weeks in order to obtain plants free from blue mold infection. When plants were at 5-9-12 leaf stage, their stems were infected at the base by injecting with a hypodermic syringe a spore suspension of Peronospora tabacina into the stem of the 2-3 rd internode. To insure the establishment of infection in the stem, plants were kept at a temperature of 20°C and humidity near saturation for 48 hours. Following this initial period of incubation, the temperature was increased to 29°C and humidity was kept below 70%. This technique caused infection of foliage and insured both plants with mild stem infection and healthy leaves. Three weeks after injection both stem injected and control plants were foliage inoculated by spraying with a conidial suspension of P. Tabacina and then transferred to a glass house where condition were favorable for leaf infection. Resistance was obtained in treated plants while controls injected with water were attacked by the disease.[11]

b) Hybridization

The method of interspecific hybridization and continued back crossing to commercial varieties with rigid selection for disease resistance was carried for quite a long time. Some disease resistant tobacco plants which could be used commercially were obtained in Australia, France, Italy and Germany. However, we see that the ionizing radiation can also help to solve some interspecific incompability problem occurring from time to time with this method. Swaminathan and Murty showed that the incompability that exists in the cross Nicotiana tabacum X N. rustica can be overcome by exposure to X rays where the incompability is due to the insufficient growth of the pollen tube. Similarly it was shown that the particle seed failure obtained from the reciprocal cross can be improved by X-irradiation of mature pollen.[12] Also the fact that radiation induces a break between genes usely produce undesirable mutation but can be useful sometimes as demonstrated by an American geneticist, Dr. Sears. While crossing wild and cultivated wheat to obtain a rust disease resistants hybrid, he was faced with the problem of obtaining many deleterious features from the wild wheat which could not be separated from the rust resistant gene. By using X rays and irradiating these hybrids, the linkage with the undesirable feature was broken and the segment of chromosome bearing the gene for disease resistant was separated, and attached to the wheat chromosomes. A variety indistinguishable from wheat but having very potent disease resistance of the wild wheat was produced.[13]

c) Producing mutants with chemicals & radiation

Mutant production with chemicals is a well known factor. For example, abnormal and retarded growth was observed if tobacco seedlings were treated with the aqueous extracts of seeds (tobacco) which had been stored previously and which had been tested for abnormal character when subjected to germination, showing natural mutation due to chemical changes during storage.[14] Chemicals such as diethyl sulfate, ethyl methansulfonate, β-propionlactone, ethylenimine are known to produce mutation through chromosomes aberration.[15] High dose of radiation are also known to be mutagenic while the application of small dose can have some beneficial results.[16] It was shown that air dried seeds of 21 agricultural crops irradiated with doses of 500-5000 r , varying according to the plant, brought an increase in germinating power, accelaration in sprouting, increase in vitamin sugar and protein content of the crop.[17] Literature shows an intensive study on the damages produced by increased doses of radiation along with the possibilities of introducing useful mutants. Although the latter problem showed some satisfactory results in recent dates as useful mutants production in Russian wheat,[18] considerable work must still be done.

A similar type of work in tobacco seems to be promising because of its allotetraploid condition and ability to tolerate part and whole chromosome deficiency. This is an advantageous characteristic in point of view of getting viable mutation in the irradiated population.[19] So, a survey of work done on mutagenic production of tobacco plants by using ionizing radiation, along with the factors influencing radiosensitivity and ways to control it, is given below:

Production of mutants from the Nicotiana species was first performed with Goodspeed in 1927. He found that effective alteration was readily induced in those species by treatment of reproductive tissues with high frequency radiation. Then mutation experiments were started on tobacco plants in 1955 at the Indian Agricultural Research Institute. Some data on mutant formation of N. tabacum obtained from seeds irradiated with different doses of X rays, U.V., Thermal neutron P_{32} and colchicine were reported in 1961.[19] A suggestion for selection work is also described in the following way:

> "Since M_1 plant will be a mosaic constituent of both affected and unaffected cell, it will be desirable to carry forward the seeds from each capsule of each M_1 plant as separate families. Since each capsule contains several hundred seeds so chances of obtaining mutations in the M_2 generation will be very low if all seeds from a plant are mixed and a small sample used for raising the M_2. The success in mutation will depend largely upon the efficiency and rigor of selection procedure adopted in the M_2 and M_3 generation and the specificity of the objective of the experiment."

In 1957, an investigation on the artificial alteration of genetic inheritance of German plants were studied. Among all kinds of mutants produced by X rays, extracts which were resistant to vein burning disease was found.[20] Some morphological irregularities in Nicotiana were produced by thermal neutrons,[21,22], while better results were reported as useful mutants induced by X rays.[23,24] Work on polygenic mutability induced by X rays were performed in Italy.[25,26] Research on morphological, biochemical and cytological modifications due to an increasing dose of radiation on Nicotiana were carried out but produce mostly damages.[27] Also work on the combined effect of X rays and chemical mutagens on Nicotiana tabacum showed that a simply additive effect was noticed if seeds irradiated with gamma rays were pretreated with ethylenimine, while if pretreated with β-propiolactone and ethyl methansulfonate under the same condition, an effect superior to the sum of the separate effects of the chemicals and Gamma rays were obtained.[28] On the other hand, recent work on the effectiveness of X irradiation of seeds in producing mutation in maize, wheat and tobacco with characteristics desirable for crop improvement was reported.[29] Also in Japan, several strain of tobacco were irradiated with X and Gamma rays and mutant useful characteristics were selected for breeding. In order to increase cross fertility, X and Gamma rays were applied on pollen grains of wild tobacco which were used for interspecific crossing with cultured tobacco to induce resistant characteristic from the wild type. It was found that 30 Kr. is a suitable dose for the introduction of mutation. Useful mutants with disease resistant properties, better yield and higher quality were obtained.[30]

A fight against the Blue Mold infection by producing resistant mutants through ionizing might be possible. However, a thorough knowledge of effects of ionizing radiation on tobacco is essential and involve various branches of science. So a report which covers effecting the radiosensitivity of seeds in general and methods used to control its effect is given below:

Radiosensitivity of seeds depends on two main factors: 1) Their environment, 2) their biological trait and chemical composition.

1) Radiosensitivity depending on the environment of seeds

Some investigators had shown experimentally by the method of electron spin resonance that the action of high energy photon of Gamma irradiation upon high polymeric substances such as lipoprotein, glycoprotein and nucleoprotein produce free radical centers. They also found that each active center requires 10-100 ev of absorbed energy. Their action are enhanced by increasing their longevity which depends on the conditions discussed below.[17,31]

a) Moisture

The (ESR) method showed that the decay of free radicals is markedly accelerated by increased moisture or temperature. Investigations were made to elucidate the sensitivity effect of extreme drying with electron spin resonance absorption. It was seen from measurement, that irradiation induced free radicals has a fairly constant amount in dry seeds, while in seeds with medium water content, free radicals soon disappears.[32]

b) Oxygen

Tests were performed on barley seeds irradiated with X rays at 4-11% moisture under various partial pressure of oxygen. They were then sprouted and observed for seedling heights as a measure of damage. Seeds irradiated with Gamma or neutron under pO_2 70 atm. gave remarkable uniformity of seedling height. Some heterogenity of growth observed after control of exposure under ambiant conditions were ascribed to variation in oxygen content of individual seed. The sensitivity of barley was increased 8-9 times by complete saturation with oxygen.[33] It is seen that the sensitizing action of oxygen is due to the conversion of long lived radicals to peroxide radicals with the action with oxygen in a chain reaction of the following type:

$$
\begin{aligned}
R^* + O_2 &\longrightarrow R\,COO^* \\
R\,COO^* + RH &\longrightarrow ROOH + R^* \\
R^* + O_2 &\longrightarrow RO - O^* \quad \text{etc.}
\end{aligned}
$$

One radical leads to the creation of hundreds of molecules of hydroperoxide until chain is broken. The development of the above reaction leads to a decrease of thymine in DNA and uridine in RNA and disturb the protein of these nucleic acids. The occurrence of the chain reaction will also bring damage or activate a number of enzymes thus changing the metabolic processes.[17]

c) Combined effect of oxygen and moisture

Moisture content of the embryo is also a critical factor for controlling the oxygen and after effect in seeds. As the moisture decreases from 3% to 1%, oxygen appear to be ineffective. The observed oxygen effect at normal atmospheric pressure seems to be maximum at 4-5% for barley seeds, then decreases with increasing moisture up to around 12-16%. It is concluded that in irradiated seeds of increasing moisture, there is an increasing competition between oxygen and water molecules for reaction with radiation induced radicals. Water radicals prevent, while oxygen radical reaction enhances post radiation induced biological damages.[34]

d) Carbon dioxide

Pretreatment with air and CO_2 increased chromosome aberration over that in control material treated in air with irradiation of 6000 r. CO_2 pretreatment was ineffective in increasing the frequency with irradiation in nitrogen or in vacuo. It therefore appears that CO_2 acts synergestically with oxygen in increasing the frequency of X ray induced aberration.[35] It was also shown that although CO_2 increases chromosome aberration, it has no effect on mutation frequency.[36]

e) Storage effect

When seeds of barley (moisture 4%) were germinated immediately after irradiation, they gave normal distribution of seedling growth about the mean. However, when stored at room temperature before germination, the seedling height about the mean becomes progressively skewed as a function of time. Pre-irradiation treatment with 75°C during a period of 24 hrs. showed a protective effect of heat treatment against a subsequent dose of X rays and persisted for at least two months between heating and irradiation, provided that humidity of the storage atmosphere did not change. On the other hand, post-irradiation treatment at 75-80°C enhanced the injury that was observed during post irradiation storage at room temperature.[37] An investigation between the relationship of moisture content of seeds and the damage from post-irradiation storage was performed. It was found that seeds of normal dryness (10%) do not change if stored under normal conditions. However, if irradiated seeds of normal dryness are stored in a desiccator before germination, the radiation induced damage increases in the post-irradiation period. Conversely, desiccated seeds show a post-irradiation recovery if stored under condition of normal moisture.[38]

2) *Radiosensitivity depending on the chemical composition and biological trait of seeds*

a) Chemical composition

Plants vary greatly in their radiosensitivity. Thus the LD_{50} of Brassica seeds is 300 times the LD_{50} for seeds of Pinus sylvetris. Observations that aqueous extracts of sensitive seeds can make resistant seeds more sensitive and vise versa, showed a relationship between their chemical constitution and their radiosensitivity. Chemical fractionation of pine seeds extract was used to show that its radiosensitizing action was caused by linoleic acid. This is thought to be due to peroxides of unsaturated fatty acids produced by irradiation.[39] Also plants containing an appreciable amount of reducing substances show more resistance against radiation.[40] Steffenson has demonstrated that calcium deficient plants have more spontaneous chromosome breakage and are more radiosensitive. Phosphat deficient plants have shown an increased mutational response to X rays, and boron enriched tissues an increased sensitivity to thermal neutrons.[41]

b) Biological trait

1) Ontogenetic development:

Seeds differing in ontogenetic development contain embryo differing in vitality. Their endosperm also differ in the amount of nutrient reserves. So ontogenetically younger seeds are more radiosensitive than ontogenetically older seeds.[42]

2) Dormancy:

Dormant seeds are more radio resistant than seeds ready to germinate. In the latter case, the seeds are easily effecting by environmental conditions including radiation treatment. Also the physiology of the seed with biochemical changes at the different stage of its formation and germination are of prime importance.[42]

3) Coat effect:

The structure of the seed coat influence the penetration of radiation. Permeability of seed coating should also be taken into consideration as it effects the passage of important external factor like oxygen to the embryo.[43]

4) Size of the embryo:

The relative size of the embryo to the seed is important. For example, if the embryo is small and located at one end of the seed, irradiation at the embryo would be more effective than exposure to the opposite end. In the latter case, radiation had to penetrate not only seed coat, but stored food and endosperm. Chemical composition of the stored food is an addition factor. On the other hand, if the embryo is large and fill the entire seed, great importance should be given to the localization of the radiation because certain points of the embryo are more vulnerable than others.[43]

5) Chromosomes:

The chromosome size is an important factor in determining the tolerance of a species. Since the chromosome size is related to he the size of the nucleus, an investigation of the relationship of the size of the nucleus and radiation dosage showed that the larger the nuclear volume the more sensitive the cell. Also the nuclear volume and the amount of DNA per nucleus are related. So the radiosensitivity of different plant species can be correlated with the average amount of DNA per nucleus. An increased chromosome number has a protective effect. Polyploidy has the same effect but can be decreased by the contrary effect due to the increase of the nuclear volume.[42]

METHODS TO INDUCE MUTATION RATES AND REDUCE RADIATION DAMAGES

There are several protection methods to reduce radiation damage. For example, low dose rate, low temperature, exclusion of oxygen. Also soaking in a solution which contains a reducing substance is proved to be effective. Ascorbic acid was used on an experiment with mulburry seeds. The reducing property of ascorbic acid hinders the action of peroxide formed in the irradiated cells.[40] Also other chemicals such as cysteine, thiourea, sodium hyposulphite and glutathione, reduce the effect of a given dose of radiation on plants. On the other hand, potassium cyanide, hydrogen peroxide and uranyl acetate are known to enhance its effect.[36]

The fact that chemical mutagen, diethylsulfate, ethyl methan sulfonate will produce higher frequency mutation than with radiation but fewer gross chromosome aberration is known. Similar results were obtained with a post-irradiation shock technique. This consist of treating seeds with gamma rays at a temperature of -78°C, then give one minute shock treatment in 60°C water. This method showed higher survival, fewer chromosome aberrations, and a higher ratio of mutation to aberration than similarly treated irradiated non shocked seeds. Seeds differing in moisture appear to respond differently, but a protective effect has been observed from both high and low moisture seeds.[44] It was also shown that pre-treatment with colchicine result in a modification of the spectrum of mutations obtained from irradiated barley seeds.[36]

Lately, an investigation on the influence of paramagnetic ions on the radiation sensitivity of plant seeds were performed. It was found that unlike diamagnetic ion, paramagnetic ions has a protective effect. This phenomena is attributed to the quenching of excited triplets following irradiation.[45]

CONCLUSION

We have seen from various literature surveys, that ionizing radiation can help in many ways problems facing 1) Hybridisation (e.g. incompability, the breaking and rearrangement of chromosomes which may result in separating a desirable gene, 2) Improvement of crop by early maturation and increase of useful chemical, 3) May produce commercially useful mutants but still in very few species (see pp. 3). So ionizing radiation should not be used only for the purpose of producing disease resistant mutant, an investigation still in an experimental basis for tobacco. Radiation can be used in conjuction with hybridization which is another way of fighting the blue mold disease.

As far as producing disease resistant mutant by ionizing radiation is concerned, one must first decide about the minimum dose that should be given to the tobacco seeds in order to obtain any change at all. Then increase the radiation while studying its effects. This can be facilitated by an estimation of the radiosensitivity of tobacco seeds, from the knowledge of their biological trait and chemical composition. Literature has some controversy as to whether there is a differential efficiency of neutrons, X and gamma rays. So there is little justification apart from the availability of sources and convenience to state a preference for one as opposed to another type of radiation.[46] Very great importance should be given to work on seeds of similar states as their ontogenetic development, dormancy, moisture and oxygen content. Environmental variables like time between irradiation and germination, storage conditions and temperature before and after irradiation, chemical composition of the soil used and planting technique should be carefully recorded. Importance should also be given to chemical mutagens which can act as a supplement to radiation for mutation research. The chance of obtaining reproducible results depends on a thorough knowledge of variable factors effecting the radiosensitivity of tobacco seeds. Unfortunately these factors are not all known, but if care would be given to keep known ones constant and work with one variable at a time, one might hope to approach ones goal quicker. This involves a teamwork between various branches of science such as agriculture, genetics, radiobiology, chemistry, biophysics.

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