Effects of Gamma Radiation in Concurrency with Certain Environmental Conditions on Lethal and Physio-chemical Responses of Penicillium expansum L.

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EFFECTS OF GAMMA RADIATION IN CONCURRENCE WITH CERTAIN ENVIRONMENTAL CONDITIONS ON LETHAL AND PHYSIO-CHEMICAL RESPONSES OF *PENICILLIUM EXPANSUM* L.

by

Tsong-Wen Chou

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

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Logan, Utah

1969
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Tsong-Wen Chou
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ABSTRACT

Effects of Gamma Radiation in Concurrency with Certain Environmental Conditions on Lethal and Physio-chemical Responses of Penicillium expansum L.

by

Tsong-Wen Chou, Doctor of Philosophy

Utah State University, 1969

Major Professor: Dr. D. K. Salunkhe
Department: Food Science and Technology

In vitro investigations were conducted to study the lethal effect of gamma radiation on the fruit spoilage fungus Penicillium expansum under certain environmental conditions, and to study some physio-chemical changes in the fungus induced by the radiation which may be related to the death of the fungus or its ability to invade fruits.

The radiation sensitivity of P. expansum conidia was influenced by such factors as nutritional condition of the post-irradiation media, age of the spores, delayed plating, heat treatment, and chemical sensitizers. Higher survival was obtained when irradiated conidia were plated on Czapek solution agar (sugar-salts medium) than on potato dextrose agar; yeast extract added in Czapek's medium hastened colony growth but reduced survival. The 6-month-old conidia were more radiation sensitive than the 1-week-old conidia. Recovery
from the radiation injuries was observed when irradiated spores were held in the suspension for several days at 23°C. No recovery was found at 1°C. Heat treatment (58°C; 4 minutes) before or after irradiation increased the radiation effect. Difolatan (10 ppm), iodoacetic acid (50 ppm), and secondary butylamine (500 ppm) were effective radiation sensitizers to *P. expansum*. The colony growths were inhibited for various periods following irradiation of the mycelia. The duration of inhibition was influenced by the dose applied and the nutrient of the medium tested. Irradiation of the Czapek solution agar slowed the initial growth of unirradiated fungus. No such effects were found with the other media tested. From these results, it can be concluded that under proper conditions, the fungus can be effectively inactivated by low doses of gamma radiation and thus retain the quality of fresh fruit with a minimum irradiation cost.

Abnormal colonies and mutants were induced by the radiation. Some mutants were no more radiation resistant than the normal strain. The culture grown from irradiated conidia produced more of pectolytic enzyme than did the control after 3 days of post-irradiation incubation. The enzyme production was stimulated by the irradiation.

Nucleic acid content of the mycelia grown from irradiated spores was related to the growth stage of the fungus and not directly influenced by the irradiation. The slowed growth rate of the irradiated fungus indirectly influenced the nucleic acid content. When isotope labeled mycelia were irradiated, no radiation induced degradation of nucleic acid was detected in the fungus
during 8 hours of post-irradiation incubation, either by chemical or isotopic analysis.
INTRODUCTION

Many millions of tons of fresh fruits and vegetables are lost each year in the world as a result of post-harvest spoilage. In the United States alone about a quarter of a billion dollars worth of fruits and vegetables was estimated to be lost annually after harvest and before reaching the consumer (U. S. Department of Agriculture, 1965). Major post-harvest deterioration is the result of the action of the microflora present on them. One of the common types of microbial spoilage in horticultural crops is caused by Penicillium species. *Penicillium expansum* is the most prevalent and destructive fungus commonly found in fruits such as apples, cherries, grapes, peaches, etc. (Raper and Thom, 1949).

Recently, the use of ionizing radiation as a method of food preservation has received considerable attention. Application of radiation to food may be achieved by either complete sterilization of all microorganisms by employing high doses or reduction of spoilage microbes by low dose pasteurization. Complete sterilization of fresh fruits and vegetables is not feasible, for being living matter, they are susceptible to radiation injury. Quality attributes such as appearance, color, aroma, flavor, texture and some nutritive values will be damaged by the treatment with sterilizing doses. Therefore, the use of ionizing radiation in the post-harvest decay control depends upon the radiation sensitivity of causal microflora compared with the radiation tolerance of the
host. For this purpose, studies elucidating the physiological and biochemical changes occurring in the microorganism following radiation are necessary to provide a basis for practical application in the field of food preservation.

Gamma irradiation of fresh fruits to inhibit or destroy the microflora and thus prolong their storage life has been reviewed by Salunkhe (1961), and Sommer and Fortlage (1966). The application of gamma radiation to inhibit the growth of *P. expansum* on apple (Beraha et al., 1957; 1961) and *in vitro* study of the fungus (Beraha et al., 1960, Sommer et al., 1964) have been reported. However, only meager information is available concerning effects of gamma radiation on *P. expansum* particularly with regard to *in vitro* study of lethality and the accompanying physiological changes caused by irradiation.

The primary objectives of this investigation were to study the lethal effects of gamma radiation on *P. expansum* as influenced by environmental factors; and to study the changes in the fungus induced by the radiation, which may be related to the death of the fungus or its ability to attack fruits. The survival curves were obtained for irradiated spores from germination percentages and colony formation. Some factors affecting lethality of irradiated conidia such as plating media, age of spores, delayed plating, magnetic treatment, heat treatment and chemical sensitizers were investigated to provide information concerning feasible conditions to control the fungus at the pasteurization doses of irradiation. Inhibition of growth of irradiated mycelia on different media was determined. Since fruits will be irradiated for pasteurization, an experiment was conducted to find out the effects of irradiated media
on the growth of fungus. The changes in characteristics of the fungus which resulted from irradiation, such as morphology, activity of pectin-hydrolyzing enzymes, and RNA and DNA contents were also investigated. Pectolytic enzymes are the main agents of the fungus to invade its host fruits. Any change in the enzyme activity could change its pathogenicity. Since the nucleic acids play an important role in the process of heredity and protein synthesis, therefore, an attempt was made to relate the nucleic acid contents and the death of the cells.
REVIEW OF LITERATURE

Use of Ionizing Radiations in Post-harvest Storage of Fruits

The existence of ionizing radiation was recognized when artificially produced radiation called X-ray was discovered by Röentgen in 1895 and natural radioactivity was discovered by Becquerel in 1896. It was found later by several investigators that there are three kinds of ionizing radiation from radioactive element, namely alpha and beta particles, and gamma rays or electromagnetic waves. These are referred to as "ionizing" radiations because they are capable of ionizing the materials they pass through. Of these ionizing radiations, alpha particles are not very useful in food processing because of the lack of sufficient penetrating ability. Only cathode rays (analogous to the beta ray, artificially accelerated electrons with higher energy), X-rays and gamma rays (similar in nature but of different origin) are of major interest in food processing.

Bactericidal and fungicidal properties of ionizing radiation were studied soon after the discovery of radioactivity. The early investigations of the biological effects of ionizing radiation on bacteria were reviewed by Duggar (1936) and on fungi by Smith (1936). Between 1896 and 1898, several investigations failed to prove bactericidal activity of X-rays. Rieder, in 1968, reported decided lethal X-ray effects upon bacteria. The following year, Pacinotti and
Porcelli succeeded in killing several species of bacteria by exposure to uranium radiation (Siu and Mandels, 1957). Pichler and Wöber in 1922 reported that X-ray treatment killed smut fungi which had infected wheat and barley seeds (Siu and Mandels, 1957). The first paper which attempted to develop a practical technology for perishable commodities was given by Brasch and Huber (1947).

The explosion of atomic bombs at Hiroshima and Nagasaki in 1945 was a human tragedy. After the war people sought peaceful uses of atomic energy, which became possible with the advances in man's knowledge of the atom. From about 1950, waste fission products were beginning to accumulate in the nuclear reactor operation; research in utilizing these radioactive products became popular. The potential use of gamma ray emitting isotopes in preservation of foods appeared promising. Since then numerous research papers related to this area have been published.

Desrosier and Rosenstock (1960) reviewed some of the technical problems involved in the irradiation of foods, including fruits; effects on microorganisms were also discussed. An excellent review concerning effects of irradiation on the quality attributes of fruits has been presented by Maxie and Abdel-Kader (1966). Investigations related to post-harvest disease control of fruits were reviewed by Clarke (1959), Salunkhe (1961), and Sommer and Fortlage (1966).

Many investigators agreed that since fresh fruits and vegetables are living matter, complete sterilization is not feasible. Undesirable damage such
as changes in color, aroma, texture, and nutritive value would result from too high radiation doses. In relation to post-harvest disease control, the radiation dose applied would be limited by the tolerance of host crops. It has been found that radiation tolerance of fruits was influenced by species, varieties, and stage of ripeness. Salunkhe (1961) stated that the radiation dose required for pasteurization also depends upon the numbers and kinds of microbial types present on a given product prior to irradiation. However, in general, he found that a dose of 200 to 300 Krad could extend significantly the storage life of fruits without much loss in quality. Desrosier and Rosenstock (1960) concluded that surface contamination of organisms would be drastically reduced or completely eliminated by the dose of 100 to 200 Krad. Maxie and Sommer (1964) concluded that textural changes would likely be the factor limiting the application of irradiation as a practical technology for fruits, and 225 Krad would be the maximum dose that most fruits could tolerate without unacceptable susceptibility to transit injury.

**Mechanism of Action of Ionizing Radiation**

The knowledge of the effects of ionizing radiation in living systems is still fragmentary. Some of the recent reports pertaining to studies of radiobiological mechanisms were by Bacq and Alexander (1961), Errera and Forsberg (1961), Harris (1961), Jenkinson (1963), Lea (1955), Romani (1966), and Tischer and Kurtz (1957).

Ionizing radiations in passing through matter lost their energy by transferring it to electrons, thus producing electrically excited or ionized
molecules. Chemical effects follow and in most events resulted in the formation of free radicals which may combine with each other or react with other chemical species of the medium. Molecular changes caused by absorbed energy produce injuries to living cells. The direct action theory states that damage is caused by direct impact of an ionizing particle or wave at a sensitive site in the cell responsible for the radiation effect. The indirect action theory states that ionization produces chemical changes, such as formation of highly reactive free radicals from water and that these react with cell constituents, resulting in cell injuries. The indirect theory allows for somewhat greater flexibility and appears better able to accommodate such observations as effects of chemicals, of changes in oxygen concentration, etc. Pomper (1965), and Tischer and Kurtz (1957) explained by combination of the two theories which retains the feature of the direct target theory of a sensitive site and the indirect theory of radiation-induced chemical events, which in turn affect sensitive sites.

The injury may be a direct damage to genetic material, producing mutations which may or may not prove lethal. Molecular changes may cause biochemical lesions which are developed or intensified by metabolic processes of the cell. Biochemical lesions may affect vital genetic synthesis resulting in a mutation, or may affect nongenetic processes and result in an altered physiology. Both results, if sufficiently severe, may also prove lethal to the cell (Sommer and Fortlage, 1966).
Dose Response Curves of Microorganisms
to Ionizing Radiation

Survival curves of microorganisms have been obtained to relate the number of survivors and the radiation doses. The shape of the dose survivor curve on a semilogarithmic paper may be either linear or sigmoid, depending upon the strain and species of organism studied, growth phase of the cells, nature of the radiation, and other factors (Rayman and Byrne, 1957).

Many bacteria and haploid strains of yeast are inactivated exponentially while polyploid yeast and some fungal spores are inactivated sigmoidally. The exponential curves of inactivation by ionizing radiation are similar to those of thermal death curves of bacteria, and are in favor of the target theory that a hit on sensitive target site may cause the death of the cell (Lea, 1955). The sigmoidal curves may be explained by the theory that a number of hits are required to cause the death of the cell, or there could be a number of sites in the cell that must be inactivated (Atwood and Norman, 1949). The mitotic stage, the ploidy and the number of nuclei per cell may play an important role in survival curve response.

There is also a third type of survival curve which is a convex shape. The inactivation of haploid yeast cells was logarithmic in part, but then "tailed" off as though a portion of the population were radio-resistant. The resistant fraction was shown to consist of cells in the process of budding (Beam et al., 1954).
Factors Influencing Radiation Destruction of Microorganisms

According to Desrosier and Rosenstock (1960), the extent to which an organism is sensitive to radiation is dependent upon the following general factors: presence of oxygen, presence of water, sporulation, species, and different strains of the species, population of organisms, atmosphere in which they are irradiated, physical state of the medium, hydrogen ion concentration, composition of medium, age of culture, type of radiation, and post-irradiation treatment.

There are many factors influencing the effects of ionizing radiation on fungus. More thorough understanding of these factors is very important in post-harvest disease control.

Effect of Plating Media

The survival of some irradiated bacteria is influenced by the post-irradiation medium composition. Stapleton et al. (1955) have shown that sensitivity of Escherichia coli B/r cell to ionizing radiation depended on the type of medium used for growth prior to irradiation as well as type of plating medium employed after irradiation. Cells cultured in nutrient broth resulted in a smaller surviving fraction when plated on a simple synthetic medium than when plated on a complete medium. However, greater survival was observed when cells were grown in minimal liquid medium before irradiation and plated afterwards on a minimal agar medium.
Alper and Gillies (1958b) reported that different plating media gave rise to widely variable viable counts, and the surviving fraction was smallest on media which were optimal for the growth of unirradiated organisms. They concluded that inhibition of colony formation was due in part to an injury which leads to imbalance in the synthetic process of the cell, so that "recovery" or "restoration" may be brought about by sub-optimal conditions of growth.

Adler and Engel (1961) reported that the X-ray sensitivity of \textit{E. coli} B/r was altered by post-irradiation plating conditions and was smaller for cells plated on a synthetic minimal medium than on a complete medium.

Lee and Sinnhuber (1965) showed that viable counts of \textit{E. coli} B on glucose-salts agar was higher than on peptone agar although the organism was grown in peptone broth.

Myasnik and Korogodin (1968) reported that survival of irradiated \textit{E. coli} B plated on various media differed greatly. High correlation was found between the survival of irradiated cells and the viability of nonirradiated cells plated on the different media of various temperatures. Their conclusion was that variation in the levels of survival of irradiated cells may be due to a selective effect of the medium. However, they suggested that both recovery effects and selective effects may be "at work."

**Effect of Age of Culture**

Radio-sensitivity of cells changes with age of culture. Stapleton (1955) showed that resistance of \textit{E. coli} increased in the lag phase of growth
to reach a maximum at the end of this phase, and increased sensitivity to irradiation during the logarithmic phase, followed by a gradual recovery of resistance in the stationary phase.

Fungal conidia of the strains of 6-month-old *Aspergillus flavus* and *Penicillium viridicatum* cultures were more susceptible to irradiation than 3-week-old conidia (Malla *et al*., 1967).

**Recovery of Microorganisms by Post-irradiation Treatments**

It has been shown that survival of microorganisms was influenced by post-irradiation treatment. The means used to enhance recovery of irradiated bacteria are incubation at sub-optimal (Stapleton *et al*., 1953) or supraoptimal (Anderson, 1951) temperature, holding in suspension before plating (Roberts and Aldous, 1949), and starvation (Alper and Gillies, 1958a). Alper and Gillies (1958b, 1960) have suggested that a common feature of these procedures is their ability to slow down metabolism after irradiation, and thus preventing death due to "unbalanced growth." With yeast, a great increase in viability was observed when plating was delayed (Patrick and Haynes, 1964; Bacchetti *et al*., 1966).

Beraha *et al*. (1961) reported recovery in germination of *Botrytis cinerea* conidia was observed when irradiated spores were kept in a suspending medium before they were plated out.

Experiments with sporangiospores of the fungus *Rhizopus stolonifer* have shown that a portion of the potentially lethal irradiation injuries was
restored to the nonlethal condition when germination was delayed for one or two days. The recovery involved metabolism, with the required energy supplied either by oxidative respiration or, if a glucose substrate was present, by anaerobic fermentation. When the germination of spores was inhibited either by a lack of a medium suitable for germination or by anaerobiosis, the number of repaired spores was greatest at a temperature near optimum for growth (Sommer and Fortlage, 1966).

Effect of Heat Treatments

Kempe (1955) and Morgan and Reed (1964) found after ionizing irradiation, bacteria became more sensitive to heat treatment, but there was no effect of prior heating on radiation inactivation. Huber et al. (1953), Kan et al. (1957) and Duggan et al. (1963), on the other hand, observed that heating before exposure increased sensitivity of bacteria to irradiation.

Sommer et al. (1967) observed radiation-heat synergistic effect on Monilinia fructicola, Botrytis cinerea, Cladosporium herbarum, Rhizopus stolonifer and Penicillium expansum. With R. stolonifer, the maximum effect occurred when irradiation was applied first. In all other species studied, the reverse sequence resulted in the greatest inactivation.

Radiosensitizing Action of Chemicals

While some chemicals are known to protect microorganisms from the lethal effects of radiation, some chemicals are reported to reduce radiation
resistance of spoilage organisms. Vitamin K₅ and related compounds (El-Tabey Shehata, 1961; Noaman et al., 1964; and Silverman et al., 1963), iodoacetamide and iodoacetic acid (Dean and Alexander, 1962; and Lee et al., 1963) and p-aminophenol (Noaman et al., 1964) have been found to increase radiolethality of bacteria. Vitamin K₅ (El-Tabey Shehata, 1961; and Silverman et al., 1963), and maleic acid (Kiga et al., 1955) sensitized yeast.

Georgopoulos et al. (1966) reported that iodoacetamide was a very effective radiosensitizer for spores of Penicillium italicum, Aureobasidium pullulans, Rhizopus nigricans and Botrytis cinerea; and 1-naphtol reduced resistance of A. pullulans and Candida tropicalis to considerably low levels.

The combined action of diphenyl and radiation was reported to effectively inhibit the growth of the citrus decay fungi Diplodia natalensis, Penicillium digitatum, Penicillium italicum and Trichoderma viride in vitro (Barkai-Golan and Kahan, 1967).

The radio-sensitizing effect of chemicals is highly desirable for radiation pasteurization of fruits and vegetables where high radiation doses may cause damage to host product.

**Effect of Irradiated Media on the Growth of Microorganisms**

Fields et al. (1960) conducted a study on fungal growth on irradiated food components to relate increased susceptibility of irradiated food to fungal decay. The growth of Aspergillus oryzae was found to be significantly better
on irradiated glucose-asparagine and glucose when these materials were present in growth media. Inhibition of growth at highest dosage \((392 \times 10^4\) rads) was observed when the fungus was grown on irradiated starch. Fields (1959) also found that irradiated Czapek Dox broth inhibited the growth of \textit{A. oryzae}.

Frey and Pollard (1966) reported that an irradiated medium caused a halt in \textit{E. coli} cell growth. Growth was restored by catalase. Irradiated medium also caused a decrease in the incorporation of thymine, proline and valine into unirradiated cells of \textit{E. coli}. Reduction of \(\beta\)-galactosidase formation was observed, but there was no degradation of DNA (Pollard et al., 1964).

**Morphological Changes of Irradiated Fungi**

Sommer and Fortlage (1966) summarized the effect of ionizing radiation on morphology of pathogenic fungi. When irradiated spores were plated on a medium for germination, the diameter of the germ tubes was often much larger than normal. Frequently grotesque swellings occurred at various places in the mycelium. The germ tubes often grew only a short distance and then rounded up at the end to produce an abnormal germinant resembling a "dumbbell;" or grew extensively and branched, then stopped development, after which death followed. The amount of germ tube development is evidently inversely related to the dose applied. Characteristically, cross walls were almost totally lacking in species that normally form regular and prominent walls in the germ tubes.
The absorption of energy causing lethal damage could be separated from death by many hours. If irradiated spores were stored in an environment not conducive to germination, the time between energy absorption and death may be extended to a number of days. During that time respiration, the production of certain enzymes, and other metabolic functions are known to proceed at a normal or an accelerated rate.

The ability of the irradiated spore of the fungus to form a colony capable of indefinite growth is lost at a much lower dose than the ability to germinate (Beraha et al., 1959a, 1959b; and Sommer et al., 1963a).

When colonies were irradiated at a dose insufficient to inactivate permanently, growth was halted temporarily (Beraha et al., 1959a, b; and Nelson et al., 1959), to be resumed after a delay which amounts to several days. It is likely that most of the mycelia have been irreversibly injured. Only certain portions of hypha may, with time, recover. Essentially normal growth then occurs from localized area (Sommer and Fortlage, 1966).

**Mutations Induced by the Irradiation**

Radiation is an agent known for its ability to cause genetic changes in biological material. Mutation production usually increases when dose increases. Irradiation treatment resulted in many mutants among surviving pathogens. Genetic changes appeared in many ways, either morphologically or physiologically.

Barron (1962) in his studies of parasexual cycle of *P. expansum* found that there were morphological mutants and nutritional mutants produced
by radiation treatment. The morphological mutants were related to the pigmentation of conidia such as white, brown, olive and pale blue. Most of the nutritional mutants were satisfied by either amino acid or vitamin supplement, and only a few responded to the nucleic acid supplement. Although an increase in dose resulted in an increase both in the numbers of abnormal colonies and mutants, the nutritional mutants in the normal-appearing survivors from the gamma-irradiated stocks never exceeded 1 percent.

A radiation resistant mutant was observed in \textit{E. coli} and studied extensively (Greenberg, 1964). Radiation resistant strains of pathogenic fungi may appear and cause some trouble when radiation pasteurization is widely applied, because they may not be killed effectively at normal pasteurization doses. Most mutants appeared to be less pathogenic than their parents (Beraha et al., 1964). Even a mutant with a greater \textit{in vitro} growth rate did not have greater pathogenicity (Buddenhagen, 1958).

\textbf{Pectolytic Enzymes of Irradiated Fungi}

Decay in plant tissue is associated with, and possibly dependent upon, maceration by hydrolysis of the insoluble pectate of the middle lamella. \textit{P. expansum} was found to produce highly active pectic enzymes in rotted apple tissue or culture filtrate (Cole and Wood, 1961).

The ability of spores of \textit{Rhizopus stolonifer} to produce pectolytic enzymes was found to be more radiation resistant than the potential for colony formation or the ability to germinate. Spores made incapable of
forming colonies by irradiation continued to produce pectolytic enzymes after a 6-day period following irradiation treatment (Sommer et al., 1963b).

**Nucleic Acids of Irradiated Microorganisms**

DNA, taking an important part in the synthesis of proteins and in the process of heredity, is the candidate for principal target of lethal radiation effects in most unicellular systems. Irradiation of DNA in aqueous solution resulted in the changes in viscosity, sedimentation, and light-scattering, indicating degradation of DNA molecules (Ginoza, 1967).

The damage of DNA could be as follows: (A) Structural damage resulting in major distortion of the DNA structure and change in its topology. Most often breakage of phosphodiester bonds with resulting single- or double-chain scission, or breakage of covalent linkage of the complementary strands, binding to protein or other macromolecular cell components, and other changes affecting large regions of DNA. (B) Change in information caused most often by more subtle radio-chemical modification of individual DNA bases. This modification could lead to (1) inconsequential changes, (2) major missense or fraudulent information and (3) complete nonsense (Szybalski, 1967).

It is generally recognized that the amount of the degradation of DNA required for a genetic effect is so little that it is probably undetectable by normal chemical analysis (Desrosier and Rosenstock, 1960 and Jenkinson, 1963).

One of the observations on radiation induced DNA degradation in
the microorganism cell was made by Stuy (1961). He observed considerable DNA degradation in *Haemophilis influenzae* following X-irradiation. The cause was considered to be the activation of cellular deoxyribonuclease. Since the breakdown was followed by an increased synthesis of DNA, he concluded that it is not an important cause of cell death. Drakulíc and Kos (1963), Miletić *et al.* (1963) demonstrated radiation induced DNA degradation in a number of strains of *E. coli*. The effect of oxygen in the pre-irradiation medium on the degradation process was demonstrated by Pollard and Achey (1966). Shaffer and McGrath (1965) found that the amount of degradation among various cells in the population was similar, not restricted to those cells destined to die. Cells heated 70°C for 10 minutes before showed no DNA degradation after irradiation (Pollard and Achey, 1966). The degradative loss of DNA is temperature dependent, which suggests that an enzymatic process is associated with degradation (McGrath *et al.* , 1966; and Achey and Pollard, 1967). The amount of DNA degradation was proportional to the dose up to a certain maximum. With regard to the relation between dose and number of survivors, it could be argued that the phenomenon of DNA degradation is central to the killing of bacteria. On the other hand, it could be argued that DNA degradation is a protective mechanism in which the cell discards a genome that is now useless because of radiation damage and preserves a genome that is intact (Achey and Pollard, 1967).

DNA synthesis was inhibited after exposure of bacteria to X-rays (Doudney, 1956 and Miletić *et al.*, 1961), RNA synthesis was depressed in
irradiated cells of *E. coli* B/r (Frampton, 1964) and part of the RNA in an irradiated culture of *E. coli* B was beginning to breakdown after 30 minutes of incubation (Pečevsky and Miletić, 1966).

**Studies on Gamma Irradiation of *P. expansum***

The application of gamma radiation to *Penicillium* species on apricots, peaches, pears, strawberries and sweet cherries to depress their growth was successfully demonstrated by Cooper and Salunkhe (1963), Dhaliwal and Salunkhe (1963), and Salunkhe (1961). *Penicillium* species were found more sensitive to radiation than other species of mold.

Beraha *et al.* (1957) reported that *P. expansum* inoculated into apples and incubated for 24 or 96 hours prior to irradiation was suppressed for 10 days at 70-75°F by 200 Krad. Beraha *et al.* (1961) reported that 50 Krad did not reduce *P. expansum* rot whereas 100 Krad reduced day-old infections and 200 Krad was required to check decay in 4-day old infections.

An *in vitro* study of gamma sensitivities of *P. expansum* along with other decay pathogens was reported by Beraha *et al.* (1960). The dose response of the selected *Prunus* fruit decay fungi to inactivation of their colony-forming potential by gamma irradiation was studied by Sommer *et al.* (1964). Sigmoidal inactivation at the low doses was observed for those fungi. Conidia of *P. expansum* were the most sensitive among the fungi tested. Synergistic effects of combined gamma radiation and heat treatment on colony-forming ability of *P. expansum* were reported by Sommer *et al.* (1967).
MATERIALS AND METHODS

Radiation Facility

The irradiator used as a radiation source (American Nuclear Corp. Oak Ridge, Tennessee, Figure 1) was loaded with 1306.8 curies of Cs\textsuperscript{137}, and was arranged in such a manner as to give an even flux of gamma radiation dose in the 30 x 29 x 10 cm irradiation chamber. The dose rate as measured by Bausch and Lomb glass rod was 345 roentgens\textsuperscript{1} per minute in January, 1966 (Sharma, 1968), and the spore suspension absorbed approximately 20 Krad\textsuperscript{2} per hour. The irradiation temperature was about 20 to 24° C.

Microorganism and Culture

A strain of Penicillium expansum, isolated from a blue mold rot in apple, was selected for the experiments. The stock culture was maintained on potato dextrose agar slants. All media were prepared from Difco dehydrated products (formula in Appendix).

\textsuperscript{1}The roentgen (r) is the quantity of electromagnetic radiation which produces an electrostatic unit of charge of either sign per cubic centimeter of dry air at 0° C and at standard pressure. Solely for exposure doses.

\textsuperscript{2}The rad is the quantity of ionizing radiation which results in the absorption of 100 ergs per gram of irradiated material at the point of interest. Solely for absorbed doses and independent of the kind of ionizing radiation used. The energy of Cs\textsuperscript{137} gamma ray is 0.66 Mev, and at this energy level water absorbs 0.97 rad from 1 roentgen of the exposure dose.
Figure 1. Gamma Irradiator (American Nuclear Corp., Oak Ridge, Tennessee). Radiation source: Cs$^{137}$. Irradiation chamber: 30 x 29 x 10 cm. Dose rate was 345 roentgen per minute in January, 1966.
**Preparation of Spore Suspension**

Conidia harvested from cultures grown on Czapek solution agar for 1–2 weeks were suspended in sterile distilled water and filtered aseptically through a layer of glass wool (about 1 cm thick) to eliminate aggregated spores and hyphae. The suspension was, except when otherwise stated, then adjusted to $10^6$ spores per ml with a Levy and Levy-Hauser improved Neubauer Ruling counting chamber.

**Germination of Irradiated Conidia**

Pyrex test tubes (1.2 x 10 cm), covered with aluminum foil (0.001 cm thick), each containing 4 ml of spore suspension were irradiated at different doses, from 20 to 350 Krad. Following irradiation, 2 ml of spore suspension was poured onto a prepared Czapek solution agar petri dish. When the spores had settled on the agar surface, the surplus supernatant was discarded. The plates were incubated for 20 to 40 hours at 23°C, then 5 randomly selected areas on each plate were observed under the low power of a microscope. Sufficient fields were counted in each area to give 100 spores and the relative germination percentage among these spores.

**Colony Formation of Irradiated Conidia**

To determine the effect of treatment on the ability of conidia to produce colonies, test tubes containing 1 ml of spore suspension were irradiated at various dose levels, then diluted with a known volume of sterile distilled
water and plated within 2 hours after irradiation on the following media: Czapek solution agar (pH 5.0 with NCl (CA)), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Sabouraud maltose agar (SMA), and nutrient agar (NA). Colonies were counted after 2 days of incubation at 23°C, except CA, where colonies were counted after 3 days of incubation. In one experiment, 0.4 percent of Difco potato extract or Difco yeast extract was added to CA, and colony counts were made. The platings were also made on CA and PDA for the comparison.

Since only a small fraction of spores formed colonies after the irradiation, the survival was expressed as a fraction, and calculated as

\[
\text{Surviving fraction} = \frac{N}{N_0}
\]

where

- \(N\) = number of colonies per ml of irradiated spore suspension
- \(N_0\) = number of colonies per ml of unirradiated control.

**Age of Spores**

To determine the effect of the age of spores on their response to irradiation, a CA culture was kept for 6 months at 23°C. Then the spore suspensions from this culture and also of 1- to 2-week-old conidia were irradiated at several doses, and the survivals were calculated as above. CA, PDA and SDA were used for plating.
Delayed Plating

To study the recovery of irradiated spores during the post-irradiation holding, irradiated spore suspensions in test tubes received doses of 100 Krad, were held at 23°C and 1°C for up to 6 days in dark incubator or refrigerator, and subsequent plate counts were made on CA, PDA and SDA.

Magnetic Treatment

The test tubes containing irradiated spore suspension were placed in an electromagnet generated magnetic field, having a distance between the two poles of 16 cm and a magnetic strength of 400 gauss. After 1 to 3 days exposure, the tubes were taken out and plate counts were made on PDA.

Heat Treatment

To study the synergistic action of heat, some spore suspensions were heat treated before or after 40 Krad irradiation. Test tubes each containing 1 ml of spore suspension were placed in a hot water bath, allowed 1 minute to rise to 58°C, held at that temperature for 4 minutes, then cooled to room temperature in a 10°C water bath. The interval between the heat treatment and the irradiation was less than 30 minutes. Plate counts were made on PDA.
Chemical Sensitizers

To determine the sensitizing action of chemicals\(^3\) on spore survival, 0.5 ml of the chemical solution, mostly fungicides, was added into a test tube containing \(10^6\) spores in 0.5 ml suspension. The suspension mixture was irradiated at 50 Krad, and the colonies were counted on PDA.

Colony Growth From Irradiated Mycelia

For the colony irradiation, three spots on each of duplicate plates of CA and PDA were inoculated with a loopful of spore suspension. After incubation at \(23^\circ\text{C}\) for 24 hours, the plates were exposed to gamma rays at several dose levels, and were again incubated at \(23^\circ\text{C}\). The colony diameters were measured periodically and compared to the control.

For the mycelial disc irradiation, a spore suspension was poured on a prepared CA plate and incubated at \(23^\circ\text{C}\) for 2 days. Discs of mycelia with 5 mm diameter and 1 mm thick were aseptically cut with a cork borer

from these plates. Discs were placed in sterile petri dishes and then irradiated. Subsequently, two discs were placed on each of triplicate plates of various agar media and were incubated at 23°C. The media used were CA, PDA, SDA, and apple juice agar (apple juice, 1 part; water, 2 parts; agar, 1.5 percent) (AJA). The colony diameters were measured periodically.

**Media Irradiation**

To determine the effects of irradiated agar media on the fungal growth, petri dishes containing 15 ml of agar medium were exposed to gamma rays at 100 and 200 Krad. AJA, CA, PDA, and SDA were irradiated. Conidia were then inoculated on the media, 2 loopfuls on each plate, and the colony growth was measured periodically.

To determine the effect of irradiated broth media, Czapek Dox broth (CDB) and potato dextrose broth (potato extract 0.4 percent, dextrose 2 percent (PDB) in separate 125 ml beakers covered with aluminum foil, each containing 80 ml, were irradiated at 20, 50, 100, 200 and 400 Krad. Twenty ml aliquots of the irradiated broth was poured into sterile 250 ml Erlenmeyer flask, 1 ml of conidia suspension containing $2 \times 10^6$ spores was pipetted into each flask, and incubated at 23°C for 5 days. Mycelia from the flasks was filtered, dried at 90°C, and weighed.
Culture for Pectolytic Enzyme and Nucleic Acid Analysis

The medium used for the pectolytic enzyme study was 0.5 percent pectin (Sunkist Pectin N, F., Sunkist Growers, Inc., Ontario, California) in CDB, adjusted to pH 5.0 with HCl before autoclaving. Pyrex glass tubes each containing 2 ml of spore suspension, $2 \times 10^6$ spores per ml, were irradiated at 50 and 100 Krad. The contents of each tube were then transferred to 50 ml of CDB medium in a 500 ml Erlenmeyer flask. The flasks in triplicate were shaken on a reciprocal shaker operating at 180 oscillations per minute with a stroke of 3.8 cm, at $23^\circ$C, for up to 7 days. Triplicate flasks were removed from the shaker each day, and the liquid cultures were filtered so that the filtrates could be employed for enzyme activity assays. After filtration, the mycelia were plunged into boiling methanol to inactivate their nuclease, freeze dried, and used to determine nucleic acids.

Assay of Pectinesterase

Four ml of a culture filtrate at about pH 5.0 and 2 ml of 1 N NaCl, were added to 10 ml of 1 percent pectin (Sunkist, NF) solution and adjusted the pH to 5.0 at $24^\circ$C. The pH of the mixture was kept at 5.0 by continually adding 0.1 N NaOH. Enzyme activity was measured based upon the amount of NaOH used in 1 hour after adding the enzyme preparation (Cole and Wood, 1961).
Assay of Polygalacturonase

Polygalacturonate-splitting enzymes were assayed using the viscosity reduction method. One ml of the culture filtrate was added to 5 ml of 1 percent pectin solution containing 0.1 M citrate buffer at pH 5.0. The reaction was carried out in a pyrex test tube (18 x 150 mm) covered with aluminum foil at 30°C for 30 minutes. The reaction was stopped by placing the tube in a boiling water bath for 4 minutes, and relative viscosity was measured in a Cannon-Fenske viscometer at 30°C.

Percentage reduction in viscosity was calculated as

\[
\frac{T_o - T_t}{T_o - T_w} \times 100, \text{ where } T_o, T_t \text{ and } T_w \text{ are flow times in seconds for the control, the test mixture and water respectively. The control was a reaction mixture containing boiled enzyme. "Pectinase" (Nutritional Biochemicals Corp., Cleveland, Ohio) was employed as the standard, using 1 mg of the powder per ml of the solution as the enzyme unit. A straight line is obtained when known enzyme concentration is plotted against percentage reduction in viscosity on logarithmic-probability paper (Cappellini, 1966).}
\]

Assay of Tissue Maceration Enzyme

Assay of tissue maceration enzyme was based on a modified pressure tester method (Sommer et al., 1963b). Tissue discs, 1.0 mm thick and 17 mm diameter from the pith of large potato tubers, were washed in distilled water, drained, immersed in 2 or more volumes of acetone, and held overnight at 0°C.
Before use, the slices were drained and soaked in a large volume of tap water for 1 to 2 hours at room temperature (20–25°C).

For each test, a total of 10 discs were immersed in a mixture of 10 ml of enzyme solution (culture filtrate which was appropriately diluted with distilled water) and 2 ml of 0.1 M citrate buffer at pH 5.0 contained in petri dishes (15 x 60 mm). After 30 minutes with occasional shaking at 24°C, discs were positioned over a hole (0.25 in. diameter), and a Chatillon fruit pressure tester (John Chatillon and Sons, New York, N. Y.) with a 1/8 inch tip and a scale range of 0 to 500 g was pressed against the slice until the tip passed through the tissue. The measurements were completed within 2 minutes after the end of the reaction.

The relation between enzyme activity and the strength of the potato tissue discs are expressed as follows:

\[ \log \left( \frac{S_0}{S} - 1 \right) = \log a + k \log (E) \]

where \( E \) is enzyme concentration; \( S_0 \) is the strength of the blank after soaking in the buffer in the absence of the enzyme; \( S \) is strength of macerated tissue, and \( a \) and \( K \) are constants. When \( \frac{S_0}{S} - 1 \) is plotted against \( E \) on logarithmic paper, a straight line is obtained (McClendon and Somers, 1960). "Pectinase" was again employed as standard, with 1 mg per ml of the enzyme considered a unit.

**Separation of RNA and DNA Fractions from Mycelium**

The preparation of the RNA and DNA fractions for nucleic acid
determinations is illustrated in Figure 2. It is a modification of the Schmidt and Thannhauser's method (Schmidt and Thannhauser, 1945; Hutchison and Munro, 1961; Mizuno, 1965; and Munro and Fleck, 1966).

**Determination of Nucleic Acids**

RNA was measured by the ultraviolet absorption. The amount of RNA was calculated from the difference between the absorptions at 260 μm and 290 μm (Minagawa et al., 1959) and expressed as percent dry weight. Yeast RNA (Nutritional Biochemicals Corp., Cleveland, Ohio) was hydrolyzed by 0.3 N KOH and used as a standard.

DNA was determined by an improved diphenylamine method (Giles and Meyers, 1965). About 1.5 percent of sulfuric acid was added to the diphenylamine reagent, because the sample was in 5 percent perchloric acid solution. Sperm deoxyribonucleic acid (Nutritional Biochemicals Corp., Cleveland, Ohio), hydrolyzed in 5 percent perchloric acid solution at 80°C for 20 minutes, was employed as a standard. DNA content was expressed as percent dry weight.

**Culture for Radioactive Nucleic Acid**

from Thymine-2-C\textsuperscript{14}

Three ml of spore suspension containing 10\textsuperscript{7} spores was inoculated into 100 ml of CDB containing 2 μc of thymine-2-C\textsuperscript{14} (New England Nuclear Corp., Boston, Massachusetts) in a 500 ml Erlenmeyer flask. After
Dry mycelium (20-40 mg)

2 ml of 5% TCA (Trichloroacetic acid)

Centrifugation (6000 rpm, 0°C)

Residue

2 ml of 5% TCA at 0°C

Centrifugation

Residue

Washing and centrifugation
3 ml of 2% Na acetate in 90% ethanol at 0°C
3 ml of 95% ethanol at 0°C
3 ml of ethanol-ether (1:1) twice, 20-25°C

Residue

Air drying
2 ml of 0.3 N KOH at 37°C for 10-16 hrs.
Cool to 0°C
0.1 ml of 6 N HCl and 0.2 ml of 60% PCA (Perchloric acid)
Cool to 0°C

Centrifugation

Residue

1.7 ml of 5% PCA

Centrifugation

Residue

2 ml of 5% PCA
Heat to 80°C for 20 min.
Cool to 0°C

Centrifugation

Residue

2 ml of 5% PCA

Centrifugation

Residue

Supernatant

RNA fraction

Supernatant

DNA fraction

Figure 2. The schematic diagram of procedure for separation of RNA and DNA from mycelium.
incubation on a shaker for one and one-half days, 10 flasks were removed, and the mycelia were collected. They were then filtered by suction and washed with unlabeled medium. The filtered mycelia were resuspended in 200 ml of unlabeled medium in a Waring blender (30 seconds at low speed twice), then made up to original volume for irradiation. All of the procedures were done aseptically. Fifty ml of culture was poured into each of several sterile 125 ml beakers covered with aluminum foil, and half of the group was irradiated with 100 Krad dose. The irradiated cultures were collected, mixed well, and a 50 ml aliquot was poured into a sterile 500 ml Erlenmeyer flask, for incubation on the shaker at 23°C. Two treated and two control flasks were removed every 2 hours, and the mycelia were filtered. They were then plunged into boiling methanol, cooled, and freeze dried after removal of the methanol.

**Determination of Labeled Nucleic Acids**

It was found that thymine-2-C\(^{14}\) was not incorporated directly into DNA as expected before the experiment, but instead, converted to other pyrimidine derivatives, and, as the result, the radioactivity was also found in RNA fraction. Therefore DNA degradation could not be determined by counting the radioactivity of whole cells. It was necessary to isolate the DNA fraction from the mycelium before counting. Approximately 30 mg of the freeze-dried mycelium was ground in a Ten Broeck type tissue grinder with approximately 3 ml of 5 percent trichloroacetic acid at 0°C, then poured into a centrifuge tube. The grinder was washed twice with 1 ml of the acid solution,
and the washings were mixed with mycelium suspension. Isolation of the RNA and the DNA fractions and chemical determination of nucleic acids were the same as described before. One ml of the RNA or DNA fraction was pipetted into a centrifuge tube, and perchloric acid was neutralized and precipitated with 50 percent KOH solution, cooled to $0^\circ$C, and then centrifuged. The precipitate was washed with 0.2 ml of distilled water and centrifuged again. The two supernatants were combined and dried in a stainless steel cupped planchet (3 cm diameter) under an infra-red lamp. Radioactivity was counted with a thin-window gas-flow counter.

**Statistical Analysis**

In order to evaluate the effect of irradiated media on growth of the fungus, the data on colony diameter and mycelial dry weight were statistically analyzed for determination of the least significant differences (LSD) described by Li (1964).
RESULTS AND DISCUSSION

Conditions Influencing the Effects of Gamma Radiation on *P. expansum*

Germination of irradiated conidia

The average percentage of spore germination on CA was plotted against irradiation dose on semilogarithmic paper in Figure 3. Almost 100 percent of the unirradiated conidia germinated on this medium, and germination percentage was reduced when irradiation dosage increased. About 62 percent germinated after 100 Krad, 32 percent after 200 Krad, and 17 percent after 300 Krad doses of gamma irradiation. The dose-germination relationship curve was sigmoid with a small shoulder at the dose lower than 50 Krad. This implies that more than a single hit is required to prevent an individual spore from germination. This result is in disagreement with that of Beraha et al. on *P. expansum* (1961) and *P. italicum* (1959b), when their raw data were plotted on semilogarithmic paper, almost a straight line was resulted. This difference may be due to the different test conditions, since their spores were irradiated in Tochinai's or Czapek's media and plated on 0.5 percent water agar.

Colony formation of irradiated conidia on different media

Dose-response. When the unirradiated conidia were plated on the media, all spores grew to visible colonies after 2 days of incubation; except
Figure 3. Germination percentage curve of gamma-irradiated conidia of *P. expansum* on Czapek solution agar.
3 days on CA. Most colonies from the irradiated spores were visible after 2 days of incubation, but some new surviving colonies appeared after several more days incubation on all the media. Hence, the colony counts were made on each plate on several successive days, to ascertain all the colonies.

The survivals on the several plating media (CA, NA, SDA, SMA, and PDA), average of 4 replications, were plotted against irradiation doses in Figure 4. Although a high percentage of spores germinated after exposure to the higher doses, most of them failed to grow further to produce colonies. At 100 Krad, while 62 percent of spores germinated, only 1.5 percent formed colonies on CA; less than 1 percent formed colonies on PDA. The germination percentage of the irradiated conidia seems not important for the control of the fungus, since only a small number of those exposed would grow further to produce rots in their hosts. This is in agreement with the work of Beraha et al. (1959a, b) and Sommer et al. (1963a) done on other fungi.

*P. expansum* was cited as a radiation sensitive species by Sommer et al. (1964, 1967). The data obtained here confirmed their results. As the conidia of *Penicillium* species are uninucleate and unicellular, they have less resistance to gamma radiation than those of multicellular-spored fungi as pointed out by Cooper and Salunkhe (1963). The dose-survival curve of spores observed in the present work was also in the sigmoid form, indicating that lethality resulted from a number of events taking place in a spore cell. This sigmoid curve was not likely obtained with the aggregated spores since the spore suspension was filtered through a glass wool layer before the irradiation.
Figure 4. Survival curves of gamma irradiated conidia of *P. expansum* plated on different media.
**Effect of plating medium.** Almost equal numbers of colonies formed from unirradiated conidia on each of 5 plating media (CA, NA, SDA, SMA and PDA). Survival from the irradiated conidia, however, varied among the media. The survival was the highest on CA and the lowest on PDA. Survivals on the other three media (NA, SDA, and SMA) were between those two media. The differences were magnified when the irradiation doses were increased.

It was expected that more colonies would grow on a natural medium than on a synthetic medium, because some auxotrophic mutant might have been induced by radiation, and might survive on a natural medium. Since CA contains only sucrose and inorganic salt, whereas PDA and NA contain complex organic products, the difference in survival on different media does not seem to be mainly due to a nutritional requirement.

In order to study the effect of nutrient on the survival of irradiated spores, 0.4 percent of the potato extract or yeast extract was added to CA, and colony counts were made. The platings (6 replications) were also made on CA and PDA for comparison (Table 1). Colony growth was faster than CA when the extra nutrients were added to this medium. The growth rate on the medium containing potato extract was the same as on the PDA; and the growth rate on the medium containing yeast extract was faster. Survival on CA medium containing yeast extract was much higher than on the medium containing potato extract. On the latter medium, survival was slightly higher but close to that observed on PDA. However, survival on CA was the highest. The growth rate increase caused by the additional nutrient resulted in the lower survival.
Table 1. Survivals of gamma-irradiated conidia of *P. expansum* on supplemented Czapek solution agar and potato dextrose agar

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth rate rank</th>
<th>Surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 K rad (x 10^-2)</td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>2</td>
<td>6.4</td>
</tr>
<tr>
<td>Czapek solution agar</td>
<td>3</td>
<td>14.1</td>
</tr>
<tr>
<td>Czapek + potato extract</td>
<td>2</td>
<td>8.9</td>
</tr>
<tr>
<td>Czapek + yeast extract</td>
<td>1</td>
<td>12.1</td>
</tr>
</tbody>
</table>

In summary, the effects of plating media were: (1) the survival was the highest on CA (sucrose-salts medium); (2) the incubation time required for the spore to grow into a visible colony on CA was slower than on the other media; and (3) addition of yeast extract or potato extract to this medium resulted in faster growth but less colony formation. These facts support the view that a slower metabolism during germination may allow part of the spores to repair the radiation injury. Faster growth condition may cause unbalanced metabolism induced by the radiation to lead to death (Alper and Gillies, 1958b, 1960; Sommer et al., 1963a).

Yeast extract, rich in kinds of amino acids, nucleotide derivatives, and vitamins, resulted in higher survival than the potato extract even though growth of colony was faster on the yeast extract containing CA. The reason may be that some factors in the yeast extract stimulate repair mechanisms in the irradiated cells, as assumed by Stapleton et al. (1953). Another possibility is that some factors in the potato extract may inhibit the repair mechanism.
Components of the growth medium for irradiated spores is apparently an important factor deciding their survival.

**Effect of pre-irradiation media.** When conidia harvested from PDA culture instead of from CA were irradiated, repeated results showed that the radio-sensitivity of spores was not changed. Furthermore, the order of survival on three media, CA > SDA > PDA was not changed. This differed from the result of Stapleton et al. (1955) that survival of *E. coli* cells on different media was influenced by the pre-irradiation medium used. Conidia are the resting reproductive cells which differ from the vegetable cells.

**Effect of age of spores**

Radio sensitivities of spore suspensions of 6-month-old culture and 1-week-old culture were compared. The average of 3 replications of the survival against radiation doses are shown in Figure 5. The order of survival on three media (CA, SDA, and PDA) was the same, but 6-month-old spores were more susceptible than 1- to 2-week-old spores to irradiation damage. The differences became larger when the radiation dose was increased. All the survival curves were in sigmoid shape. The older spores were more easily inactivated by the radiation. This is in agreement with Malla et al. (1967).

Although fungal spores are resting reproductive cells, their respiration does not completely cease. Certain metabolic processes function at very low rates until the spores finally lose their viability during senescence. Asexual spores such as conidia are more short-lived than sexual spores (Cochrane, 1958; Sussman and Halvorson, 1966). The relatively high radio-sensitivity of
Figure 5. Effect of conidia age on survival curves of gamma-irradiated conidia of *P. expansum* plated on three different media.
6-month-old conidia may be due to metabolic destruction of internal substances that are protective to the radiation sensitive site of the cell or due to lessening the ability to produce certain enzymes, including "repair enzymes," which are needed for the spore to retain its viability.

**Recovery of delayed plating**

More colonies developed from irradiated conidia when the plating was delayed for several days than when plated immediately after the irradiation and were influenced by the temperature at which the spores were held.

The survival of 100 Krad irradiated conidia on three plating media (CA, SDA and PDA), average of 3 replications, were plotted against the days of holding before plating, as in Figure 6 (1- to 2-week-old conidia) and Figure 7 (6-month-old conidia). Survival increased with time of holding at 23°C. After 6 days of holding, survivors were several times more numerous than when plated immediately after the irradiation. Both 1-week-old conidia and 6-month-old conidia demonstrated the same tendency of increase in survival; the final survival ratio of 6-month-old spores was still lower than that of the 1-week-old spores.

Sommer *et al.* (1963a) showed that the manifestation of irradiation injuries by sporangiospores of *Rhizopus stolonifer* can be reduced if germination is delayed for 1 or 2 days. The same repair mechanism may be operating in the irradiated *P. expansum* conidia. Difference in the survival between the three plating media was smaller after 6 days of holding; the presumed lethal damage caused by faster and unbalanced growth on the PDA may be reduced
Figure 6. Recovery of 100 Krad gamma-irradiated *P. expansum* conidia of 1-week-old culture, held in suspension at 23°C and 1°C before plating.

Figure 7. Recovery of 100 Krad gamma-irradiated *P. expansum* conidia of 6-month-old culture, held in suspension at 23°C and 1°C before plating.
by the recovery action of delayed plating. However, the delay effect was not observed when the spore suspension was held at 1°C. This temperature dependence may be because enzymes were related to the repair mechanism. These repair enzymes are active in the dark, since the spore suspension was held in the dark incubator.

The recovery of the spores after irradiation could reduce the effectiveness of radiation for post-harvest disease control. The temperature dependence of recovery is in favor of the combination of the radiation and the low temperature storage of host fruits. Additional studies are necessary on the conditions for avoiding recovery for the effective use of gamma radiation.

Effect of magnetic treatment

When the irradiated spores were held in suspension and exposed to a magnetic field for 1 to 4 days before plating, there was an increase compared to the control in the number of colonies formed due to the recovery of spores. But no direct effect of the magnetic force on colony formation of both unirradiated and irradiated spores was found. Magnetic force was reported to inhibit the growth of bacteria (Gerencser et al., 1962), but 400 gauss of flux density used in this study may not be strong enough to alter the survival of the irradiated spores.

Effect of heat treatment

From the data of the survivals for radiation only and heat only in Table 2, survival of about $1.8 \times 10^{-3}$ would be expected if two treatments
Table 2. Survival of *P. expansum* conidia after heat treatment and gamma-irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation only (40 Krad)</td>
<td>$1.69 \times 10^{-1}$</td>
</tr>
<tr>
<td>Heat only ($58^\circ$C, 4 min)</td>
<td>$1.08 \times 10^{-2}$</td>
</tr>
<tr>
<td>Irradiation $\rightarrow$ Heat</td>
<td>$1.08 \times 10^{-3}$</td>
</tr>
<tr>
<td>Heat $\rightarrow$ Irradiation</td>
<td>$0.75 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

were additive in effect; actual results were lower than the expected value. The radiation inactivation of conidia was slightly enhanced by the heat treatment. The heat-radiation combination produced lower survival than the radiation-heat combination. This concurs with the results of Sommer et al. (1967). Heat treatment prior to the irradiation may cause denaturation of some cell components such as DNA and cell protein to become more sensitive to the radiation injury, or may cause inactivation of repair enzymes and inhibit the recovery.

The interval between the irradiation and the heat treatment was less than half an hour. It is unlikely, from Figure 6, that repair process took place during this period. Radiation treatment may cause damage to some cell components and reduce the heat resistance of the survival spores.

Post-harvest diseases could be effectively controlled by a proper combination of heating and radiation. The studies elucidating sensitivity of the fungus, as well as the tolerance of fruits, to the combination treatment are necessary.
Effect of sensitizing chemicals

The survivals of conidia irradiated in combination with chemicals are tabulated in Table 3. The fungal spores remained in contact with the chemicals for about 3 to 4 hours because of the low dose rate of the available radiation source. Many of the 18 chemicals tested, which are known as fungicides, did not reduce colony formation of either irradiated or unirradiated conidia of *P. expansum* under the test conditions. Some chemicals demonstrated fungicidal action but no sensitizing effect. Difolatin, iodoacetic acid, and secondary butylamine had significant sensitizing effects. About half of unirradiated spores lost their ability to form colonies at 10 ppm of Difolatan alone, but when irradiated in combination with the chemical survival was reduced to less than 0.1 percent of the irradiated control. A treatment with 50 ppm of iodoacetic acid produced the same results. Fungicidal and sensitizing actions were also demonstrated by secondary butylamine at 250 and 500 ppm. The 1-naphthol, Neomycin, and vitamin K₅ treatment also slightly increased the inhibiting effect of radiation, but their sensitizing effects were not so great as the aforementioned chemicals.

Sensitizing actions of iodoacetic acid and 1-naphthol has been reported on fungi and yeasts (Georgopoulos *et al.*, 1966), and of vitamin K₅ on bacteria and yeasts (Silverman *et al.*, 1963). The effective fungicidal action and the high sensitizing effect of Difolatan (*N-(1,1,2,2-tetrachloroethylsulfenyl)-cis-Δ⁴-cyclohexene-1,2-dicarboximide*) on the test fungus are noteworthy.

Secondary butylamine was found to be effective in control of *Penicillium* decay
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration ppm</th>
<th>Unirradiated percent survival</th>
<th>Irradiated 50 Krad relative survival ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Captan</td>
<td>500</td>
<td>90</td>
<td>.95</td>
</tr>
<tr>
<td>DAC 2787</td>
<td>500</td>
<td>79</td>
<td>.90</td>
</tr>
<tr>
<td>DHA-S</td>
<td>500</td>
<td>50</td>
<td>.71</td>
</tr>
<tr>
<td>Difolatan</td>
<td>5</td>
<td>58</td>
<td>.15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51</td>
<td>.001</td>
</tr>
<tr>
<td>Dowicide A</td>
<td>250</td>
<td>84</td>
<td>.86</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>23</td>
<td>.28</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>50</td>
<td>48</td>
<td>.001</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>500</td>
<td>92</td>
<td>.94</td>
</tr>
<tr>
<td>Mycostatin</td>
<td>500</td>
<td>85</td>
<td>.91</td>
</tr>
<tr>
<td>Myprozine</td>
<td>500</td>
<td>82</td>
<td>1.09</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>50</td>
<td>88</td>
<td>.69</td>
</tr>
<tr>
<td>Neomycin</td>
<td>500</td>
<td>72</td>
<td>.56</td>
</tr>
<tr>
<td>Phaltan</td>
<td>500</td>
<td>84</td>
<td>.89</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>500</td>
<td>99</td>
<td>1.03</td>
</tr>
<tr>
<td>Sec. Butylamine</td>
<td>250</td>
<td>65</td>
<td>.11</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>15</td>
<td>.01</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>500</td>
<td>77</td>
<td>.78</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td>500</td>
<td>86</td>
<td>.82</td>
</tr>
<tr>
<td>U-2069</td>
<td>500</td>
<td>90</td>
<td>.91</td>
</tr>
<tr>
<td>Vitamin K$_5$</td>
<td>30</td>
<td>58</td>
<td>.29</td>
</tr>
</tbody>
</table>
of citrus fruits (Eckert and Kolbezen, 1962), and also is a promising sensitizer.

The fungus could be effectively inactivated by combining these chemicals with low doses of gamma irradiation. Adequate combination for the practical use and the mechanism of sensitizing action of these chemicals need to be investigated.

Growth of irradiated mycelia

The average diameters of 12 colonies that were irradiated 1 day after inoculation on CA and on PDA were plotted against days of incubation in Figures 8 and 9, respectively. Growth was retarded by the irradiation, but eventually the colonies started to grow at about the same growth rate as the control. Some cells in the colonies survived even after being irradiated at 500 Krad. The periods of inhibition were increased when irradiation doses were increased.

The colony growth (average of 18 colonies) after irradiated mycelial discs were inoculated on unirradiated media are shown in Table 4. The growth rates of unirradiated colonies are in the following order: AJA > PDA > CA and SDA. Colonies from the irradiated mycelial discs did not grow for a time, but then started to grow. The periods of delay were directly proportional to the radiation dose applied. The recovery of colonies from the radiation injuries was faster on SDA and on AJA than on PDA and CA. Periods of delay were also affected by components of the media; some components may stimulate or inhibit the recovery of the cell.
Figure 8. Growth of gamma-irradiated *P. expansum* colonies on Czapek solution agar. Irradiated after 1 day of incubation.

Figure 9. Growth of gamma-irradiated *P. expansum* colonies on potato dextrose agar. Irradiated after 1 day of incubation.
Table 4. Growth of *P. expansum* colonies from gamma-irradiated mycelial discs on different media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dose Krad</th>
<th>Days of incubation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diameter in cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple juice agar</td>
<td>0</td>
<td>1.18</td>
<td>2.32</td>
<td>3.59</td>
<td>4.93</td>
<td>6.15</td>
<td>7.32</td>
<td>7.32</td>
<td>7.32</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.95</td>
<td>2.02</td>
<td>3.23</td>
<td>4.53</td>
<td>5.77</td>
<td>6.98</td>
<td>6.98</td>
<td>6.98</td>
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<tr>
<td></td>
<td>160</td>
<td>0.68</td>
<td>1.58</td>
<td>2.79</td>
<td>3.99</td>
<td>5.26</td>
<td>6.49</td>
<td>6.49</td>
<td>6.49</td>
</tr>
<tr>
<td>Czapek solution agar</td>
<td>0</td>
<td>0.94</td>
<td>1.63</td>
<td>2.15</td>
<td>2.63</td>
<td>2.92</td>
<td>3.24</td>
<td>3.47</td>
<td>3.47</td>
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<tr>
<td></td>
<td>80</td>
<td>0.66</td>
<td>1.29</td>
<td>1.93</td>
<td>2.41</td>
<td>2.76</td>
<td>3.01</td>
<td>3.25</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>0.56</td>
<td>0.98</td>
<td>1.62</td>
<td>2.13</td>
<td>2.55</td>
<td>2.88</td>
<td>3.02</td>
<td>3.02</td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>0</td>
<td>0.86</td>
<td>1.57</td>
<td>2.30</td>
<td>3.07</td>
<td>3.69</td>
<td>4.35</td>
<td>5.02</td>
<td>5.02</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.62</td>
<td>1.22</td>
<td>1.85</td>
<td>2.53</td>
<td>3.23</td>
<td>3.85</td>
<td>4.45</td>
<td>4.45</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>0.54</td>
<td>0.98</td>
<td>1.55</td>
<td>2.21</td>
<td>2.85</td>
<td>3.50</td>
<td>4.09</td>
<td>4.09</td>
</tr>
<tr>
<td>Subouraud dextrose agar</td>
<td>0</td>
<td>0.89</td>
<td>1.45</td>
<td>1.99</td>
<td>2.49</td>
<td>2.89</td>
<td>3.18</td>
<td>3.45</td>
<td>3.45</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.75</td>
<td>1.34</td>
<td>1.85</td>
<td>2.37</td>
<td>2.78</td>
<td>3.08</td>
<td>3.35</td>
<td>3.35</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>0.65</td>
<td>1.12</td>
<td>1.65</td>
<td>2.21</td>
<td>2.64</td>
<td>2.96</td>
<td>3.20</td>
<td>3.20</td>
</tr>
</tbody>
</table>
The temporal halt of the colony growth would be important in the storage of some fruits such as cherries, strawberries, etc. since their relatively short storage life would be lengthened by the irradiation.

**Growth of the fungus on irradiated media**

The diameters of the colonies grown on the irradiated media, average of 8 replications, are shown in Table 5. There were no significant differences between the growth of the fungus on the control and the irradiated media of AJA, PDA, and SDA. But growth of the fungus was slower on the irradiated CA than the unirradiated medium for the first 3 to 4 days of incubation. The microscopic observations at 24 hours of incubation showed the mycelia were longer and more branched on the control medium than on the irradiated medium. The development of colonies on the irradiated medium was slower than that of the control up to 3 or 4 days. Thereafter the growth rates were the same as that of control. Some unknown components induced in the medium by the irradiation may inhibit the fungus at the initial growth stage.

The dry weights of mycelia after 5 days incubation in irradiated broth media are shown in Table 6. The variance within treatment is small in PDA but very large in CDB. However, the data failed to show significant differences among the treatments.

*Aspergillus oryzae* was inhibited on CDB, either irradiated in liquid form or powder form (Fields, 1959). The effect of irradiated CDB on the growth of *P. expansum*, if any, was concealed by the large variance of the mycelial dry weight. However irradiated CA plate did influence the initial
Table 5. Growth of *P. expansum* colonies grown on gamma-irradiated agar media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dose Krad</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple juice agar</td>
<td>0</td>
<td>1.94</td>
<td>4.36</td>
<td>6.79</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.93</td>
<td>4.31</td>
<td>6.81</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.88</td>
<td>4.30</td>
<td>6.74</td>
<td>---</td>
</tr>
<tr>
<td>Czapek solution agar</td>
<td>0</td>
<td>0.99</td>
<td>2.47</td>
<td>3.46</td>
<td>3.94</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.88</td>
<td>2.34</td>
<td>3.31</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.74</td>
<td>2.20</td>
<td>3.30</td>
<td>3.78</td>
</tr>
<tr>
<td>Potato dextrose agar</td>
<td>0</td>
<td>1.08</td>
<td>2.55</td>
<td>3.91</td>
<td>4.91</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.01</td>
<td>2.51</td>
<td>3.91</td>
<td>4.89</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.99</td>
<td>2.59</td>
<td>3.97</td>
<td>4.96</td>
</tr>
<tr>
<td>Sabouraud dextrose agar</td>
<td>0</td>
<td>1.08</td>
<td>2.42</td>
<td>3.29</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<td></td>
<td>200</td>
<td>1.10</td>
<td>2.38</td>
<td>3.33</td>
<td>3.75</td>
</tr>
</tbody>
</table>

LSD 0.05 for apple juice agar, Czapek solution agar, potato dextrose agar and Sabouraud dextrose agar at 5th day are 0.138, 0.102, 0.098, and 0.085 respectively. LSD 0.01 for Czapek solution agar is 0.139.
Table 6. Dry weights of *P. expansum* grown in gamma-irradiated broth media, after 5 days of incubation

<table>
<thead>
<tr>
<th>Dose (Krad)</th>
<th>Czapek Dox mg/20 ml</th>
<th>Potato dextrose mg/20 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.9</td>
<td>40.3</td>
</tr>
<tr>
<td>20</td>
<td>31.7</td>
<td>40.8</td>
</tr>
<tr>
<td>50</td>
<td>36.0</td>
<td>40.6</td>
</tr>
<tr>
<td>100</td>
<td>31.6</td>
<td>39.2</td>
</tr>
<tr>
<td>200</td>
<td>33.5</td>
<td>40.2</td>
</tr>
<tr>
<td>400</td>
<td>27.5</td>
<td>41.0</td>
</tr>
</tbody>
</table>

LSD 0.05 for Czapek dox broth and potato dextrose agar are 15.9 and 3.5, respectively.

The growth of the fungus. The nature and the formation of inhibiting substance(s) is not clear at this time. Since the fruits will be irradiated for pasteurization, the effect of irradiated substrate on the growth of fungi must be considered. More studies are needed to relate the changes in irradiated substrate and the fungal growth.

**Effects of Gamma Radiation on Morphological and Selected Biochemical Changes in *P. expansum***

**Morphological changes**

A large number of the irradiated conidia germinated when incubated on the agar media. But most of the germ tubes grew only a short distance, then
development stopped. The length of the dead germ tube differed from spore to spore; some grew extensively and some even branched, but the average length was reduced when the radiation dose increased.

Most of the inactivated germ tubes appeared microscopically similar to the unirradiated fungus. Grotesque swelling of the spores or mycelia was found in only a few, and the diameter of the swellings was not wider than twice its diameter of the unirradiated fungus. Cross walls were observed.

When plates were made from the irradiated spores, colony growth was variable. Although most of the colonies had appeared after 2 days of incubation, additional ones appeared after 3 days or 4 days of incubation.

Some abnormal colony types, such as a smaller size colony, a dense hyphal colony, a sparse sporulation colony, and colonies of white or lighter green spores were noted on plates of gamma-irradiated conidia. A majority of the abnormal colonies were related to the color of the conidia. Examples of the plates are shown in Figure 10. Increasing the gamma radiation dose increased the number of abnormal colonies. Strains with the white or light green conidia were stable mutants, since they retained their color after several conidial transfers. According to Barron (1962), colors of the conidia of mutants of P. expansum were governed by the genotype of the nucleus (such as white conidia or brown conidia) or by a diffusible component(s) from the parental cytoplasm (such as olive conidia). Table 1 shows higher survival rates on CA than on yeast extract supplemented medium, indicating that auxotrophic mutants constitute a very low percentage among the survivors.
Figure 10. Colonies of \textit{P. expansum} formed from gamma-irradiated conidia on plating media.

CA: Czapek solution agar.
SDA: Sabouraud dextrose agar
1: Unirradiated conidia
2: Irradiated conidia, 150 Krad.
Two strains of mutants, with white spores and with light green spores are shown in Figure 11. When plated on PDA after 80 Krad irradiation, the survival for dark green normal, white and light green mutant strains were $0.98 \times 10^{-2}$, $1.02 \times 10^{-2}$ and $0.87 \times 10^{-2}$ respectively. The two mutants were no more resistant to the gamma irradiation than the dark green wild type strain.

When irradiated discs were inoculated on new media, the colony edges were uneven, as compared to the round circle of a normal colony, for the first 3 to 4 days of incubation. This could be due to difference in recovery rate of the injured cells in a colony or uneven distribution of viable cells in a colony. Brown colored substances were secreted around the part of the colony on the irradiated disc after a few days of incubation. This might be the metabolic or dissolution products of injured or dead cells, since this brown pigment was also observed around a normal colony if the incubation temperature was higher than optimum or if the colony was aged.

Pectolytic enzymes of the broth culture from irradiated conidia

*P. expansum* produced active enzymes which macerated potato discs and hydrolyzed citrus pectin, but pectinesterase activity was low in the fungus in CDB either with or without 0.5 percent pectin. It appears, therefore, that the fungus produced polymethylgalacturonase, which can hydrolyze pectin directly without de-esterification of pectin to pectic acid by pectinesterase. This concurs with the results of Cole and Wood (1961) that all
Figure 11. Mutants isolated from the colonies of gamma-irradiated conidia of *P. expansum*.
CA: Czapek solution agar  
PDA: Potato dextrose agar  
1: Normal (wild) strain  
2: White conidia mutant  
3: Light green conidia mutant
three groups of the enzymes were found in the juice obtained from *P. expansum* rotted apples, but no pectinesterase was found in a synthetic medium culture; the culture filtrate hydrolyzed pectin but not polypectate.

Broth culture of the fungus grown from conidia irradiated at 50 and 100 Krad and their respective controls were filtered; and mycelial dry weight, polygalacturonase activity, and potato tissue maceration enzyme activity were determined. The results of three replications are shown in Figure 12. Growth of the fungus was in the stationary phase after 7 days of shaking. The mycelia of the irradiated conidia grew slower than the control. The mycelial growth was retarded by the radiation treatment. Excretion of a water soluble dark brown colored substance was observed after 3 days of incubation in the control culture and the next day in the 50 Krad irradiated culture.

The polygalacturonase activity was measured by the loss in viscosity of pectin solution. Activity of the control cultures increased proportionally to dry weight during the first 3 days of incubation, then decreased even though mycelial dry weights increased. The enzyme activity of the culture from irradiated spores was initially lower and proportional to the mycelial dry weight but became higher than the control after 3 days of incubation, even though its mycelial dry weight was lower than that of the control. Enzyme activities of irradiated samples peaked after 4 days of incubation. The 100 Krad irradiated culture was having higher activity than the 50 Krad irradiated culture, and both were higher than the control. Apparently the production of pectin hydrolyzing enzymes of the fungus was stimulated by the radiation treatment of the conidia.
Figure 12. Activities of pectolytic enzymes in pectin Czapek Dox broth culture inoculated from gamma-irradiated conidia of *P. expansum*. 
The activity of the enzymes which could macerate the potato tissue exhibited the same tendency as that of polygalacturonase, Figure 12, except the peak of activity of the control and the irradiated culture was reached after 4 days of incubation. Enzyme activity was proportional to the mycelial dry weight in the first two days of incubation, then activities of the irradiated culture became higher than those of the control. The potato maceration enzyme macerates plant tissue by destruction of the middle lamella, the cementing material between cells. It includes several kinds of pectolytic enzymes and others such as cellulolytic enzymes (Wood, 1960). The cellulase activity was not found in *P. expansum* (Cole and Wood, 1961), so the tissue maceration activity of this fungus is mainly related to the pectolytic enzymes.

Sommer *et al.* (1963b) reported that sporangiospores of *R. stolonifer* can synthesize abundant enzymes capable of macerating plant tissue, even after the loss (through irradiation) of their ability to form colonies. Increase in enzymatic activity has frequently been seen in organisms soon after irradiation, and a suggested explanation is that the radiation broke down internal barriers within the cell and released the enzyme (Alexander and Bacq, 1961 and Jenkinson, 1963). Pectolytic enzymes of *P. expansum* in this experiment were, however, very low in the spores, and the stimulation of enzyme production was observed after more than two days of incubation. Some unknown substances may have been induced by the radiation, either in the nucleus or in the cytoplasm, and may be responsible for the stimulation of enzyme production; or some mutant may be induced among the survivors and produce large
amounts of the enzymes or excrete some compounds which stimulate the enzyme production.

Since pectolytic enzymes are important agents in breaking down the host cell wall barrier, an enhanced enzyme activity could increase the fungal pathogenicity and cause practical handicaps when radiation is applied to control post-harvest diseases. An in vivo study of the enzyme production by irradiated fungus seems necessary because of the difference in nature of P. expansum enzyme production on the rotted apple and in the synthetic medium as reported by Cole and Wood (1961).

**Nucleic acid content of mycelium from irradiated conidia**

The ultraviolet absorption curve of the RNA fraction obtained from mycelia was compared with that of yeast RNA hydrolyzate in Figure 13. The RNA content of the control mycelium in shaken CDB culture was higher in the initial stage due to the high protein synthesis activity of the cell division, and decreased during incubation (Figure 14). The RNA content of the mycelium grown from the irradiated spores was slightly lower than the control in the initial stage and became higher after 1.5 days and 2 days of incubation for 50 Krad and 100 Krad irradiated conidia, respectively, and then decreased. The RNA content of the culture of irradiated spores remained higher than that of the control, then decreased to the same level of the control. Because RNA content of the fungus changed with the growth stage, when the RNA content of a dry weight of irradiated fungus was compared to the same dry weight of
Figure 13. Ultraviolet absorption spectra of RNA of *P. expansum* mycelium and yeast RNA.
A: *P. expansum* mycelial RNA fraction in 5 percent perchloric acid.
B: Yeast RNA (Nutritional Biochemical Corp., Cleveland, Ohio) hydrolyzed with 0.3 N KOH then measured in 5 percent perchloric acid.
Figure 14. Nucleic acid contents of *P. expansum* mycelia from gamma-irradiated conidia incubated in shaken Czapek Dox broth.
the control, they were almost the same. This relation can be seen more easily from Figure 15. The RNA content was related to the growth stage of the fungus and was not directly influenced by the irradiation.

The DNA content of the mycelia was about 0.33 percent dry weight in the beginning, decreased from 2 days to 4 days of incubation, then remained at about 0.2 percent. The DNA content of the culture from irradiated spores was also at the same level in the beginning, but higher than the control at 2 to 4 days of incubation, then declined and reached the same level of the control after 5 days of incubation. The DNA content of mycelia from the irradiated spores was almost the same as of the same dry weight growth stage of the control. DNA contents of mycelia was also related to the growth stage of the fungus. The radiation treatment indirectly influenced the DNA and RNA contents by repressing the fungus growth.

Nucleic acids from irradiated mycelium

Radioactive thymine-2-\textsuperscript{14}C is widely used as a tracer for cell DNA studies in many organisms. When a small amount of thymine-2-\textsuperscript{14}C was added into CDB, and \textit{P. expansum} grown, radioactivity was detected in the mycelium. But later when the mycelium was analyzed, the radioactivity was found both in the RNA fraction and the DNA fraction, and not in the remaining protein fraction. Radioactivity was much higher in RNA fraction than in DNA fraction. This result was similar to the finding of Fink and Fink (1962), who reported that \textit{Neurospora crassa} failed to utilize exogenous thymidine directly by phosphorylation and incorporation into DNA. But apparently the fungus utilized
Figure 15. Relation of RNA content and dry weight of mycelia grown from gamma-irradiated conidia of *P. expansum*. 

0 Krad

50 Krad

100 Krad
thymidine by converting it to uridine or some related RNA precursors, which can follow the metabolic pathways of the usual RNA precursors, and eventual incorporation into DNA as a thymidylate residue. The radioactivity was higher in RNA than in DNA because RNA content was higher in the cells of the fungus. As a result, the radioactivity determination had to be conducted after the RNA and DNA fractions were separated chemically.

The results of the analysis of nucleic acid contents and radioactivity from the fungus of shaken broth cultures of irradiated mycelia are shown in Table 7. The growth of fungus was halted during and after irradiation while the fungus was transferred to a new non-labeled medium and when the shaking was temporarily stopped during the irradiation. Growth of the control was started after 4 hours of incubation and that of the irradiated mycelium after 8 hours of incubation. Although slight decreases in DNA and RNA contents were observed in both control and irradiated mycelia, no radiation-induced degradation of DNA or RNA was detected by chemical analysis or by radioactive counting during the incubation. The breakdown of DNA and RNA was found in bacteria after irradiation (Stuy, 1960; Pečevsky and Miletic, 1966; and Achey and Pollard, 1967) but has not been reported in fungi. In the lethally irradiated mouse leukemic cells, DNA degradation process was not detected from post-irradiation tissue culture (Watanabe and Okada, 1968). The degradation in bacteria was explained as an enzymatic reaction induced by the radiation. It may be associated with the killing effect of the irradiation or the repair action of the injured cell. The dose (100 Krad) applied in this
Table 7. Radioactive counts and DNA, RNA contents of gamma-irradiated mycelia labeled with $^{14}C$-thymine. The mycelia were harvested from shaken 50 ml Czapek Dox broth culture.

<table>
<thead>
<tr>
<th>Incubation time after irradiation hour</th>
<th>Dose Krad</th>
<th>Mycelial dry weight mg/50 ml</th>
<th>DNA fraction DNA content %</th>
<th>Radio-activity count/min.</th>
<th>RNA fraction RNA content %</th>
<th>Radio-activity count/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>34.0</td>
<td>.334</td>
<td>4040</td>
<td>6.62</td>
<td>29400</td>
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<tr>
<td></td>
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<td>.320</td>
<td>3870</td>
<td>6.43</td>
<td>27250</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>33.8</td>
<td>.330</td>
<td>3585</td>
<td>6.52</td>
<td>30250</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>35.2</td>
<td>.325</td>
<td>4102</td>
<td>6.52</td>
<td>33500</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>36.4</td>
<td>.322</td>
<td>3094</td>
<td>6.32</td>
<td>23680</td>
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<tr>
<td></td>
<td>100</td>
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<td>3495</td>
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<td>25380</td>
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<tr>
<td>6</td>
<td>0</td>
<td>43.8</td>
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<td>.313</td>
<td>2361</td>
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<td>8</td>
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<td>3055</td>
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</tr>
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<td></td>
<td>100</td>
<td>40.6</td>
<td>.286</td>
<td>3102</td>
<td>6.14</td>
<td>26980</td>
</tr>
</tbody>
</table>
experiment reduced the colony forming ability of the conidia to 1 percent of the control, but had no detectable effect on the nucleic acid contents. The difference between this fungus and the bacterium *E. coli* may be explained on the basis of differences in enzyme activities and cell structures, such as nuclear membrane, nucleoprotein etc. The fungal cells are more complicated than the bacteria cells. The nucleic acid of the fungal cells may be structurally more resistant to the degradation than that of the bacterial cell. Comparable enzyme may not be induced or activated, or they may not be able to penetrate to the nucleic acid site.
SUMMARY AND CONCLUSIONS

The use of ionizing radiations is a promising and successful method for prolonging the shelf-life of certain foods by killing or inhibiting the spoilage-inducing microorganisms. For post-harvest storage of fresh fruits and vegetables, the doses employed will be limited by the radiation tolerance of the host. For this reason, research has been conducted to define adequate pasteurization conditions which effectively inactivate pathogenic microflora by low doses of gamma irradiation. *Penicillium expansum* is the most prevalent and most destructive fungus found in spoiled apples. It is also the causal agent of the "blue mold rot" of many other fruits such as cherries, grapes, and peaches. *In vitro* investigations were made to study some factors influencing inactivation of *P. expansum* at low doses of gamma radiation and physio-chemical changes in the fungus induced by the irradiation which may be related to the death of the fungus or its ability to invade fruits.

**Conditions Influencing the Effect of Gamma Radiation on**

*P. expansum*

*P. expansum* is one of the radiation sensitive fungi. Although quite a high percentage of spores germinated after irradiation, only a small number of them were capable of forming colonies; almost 99 percent were inactivated when irradiated at 100 Krad. Effect of irradiation on preventing colony
formation is so great that the germination percentage of irradiated spores is of no practical significance.

The number of colonies formed from a given dose of radiation varied according to the kind of plating media. The nutritional condition influenced survival, but the medium richest in nutrient did not give maximum survival. From the results it was found that (1) survival of treated conidia was the highest in CA among the plating media, (2) growth of the colonies was the slowest on CA, (3) additional nutrient such as yeast extract added to CA hastened colony growth but reduced the number surviving, and (4) CA also provided the highest surviving when conidia from PDA were irradiated. It was concluded that slow metabolism during germination was the factor which allowed more spores to be repaired from the radiation injuries. The conditions which support faster growth may cause radiation induced unbalanced metabolism to lead to death.

The recovery of the spores of *P. expansum* was observed when the irradiated spore suspension was held at 23°C for several days before plating, probably by the same repair mechanism. The difference in degree of survival among three media became smaller after 6 days of holding. Recovery action is temperature dependent, since no increase in survival was observed after 6 days of holding at 1°C, indicating a possible enzymatic metabolism relationship.

The 6-month-old spores showed less resistance to irradiation than 1-week-old spores. The age of spore was one of the factors which influence
the sensitivity of fungus to the radiation. A magnetic field under a flux density of 400 gauss did not affect the survival of irradiated spores.

The combination of heat treatment followed by radiation demonstrated a synergistic effect. Difolatan and secondary butylamine were found to be two promising radio-sensitizers in addition to the already known iodoacetic acid, 1-naphthol, and vitamin K₅. The use of chemical sensitizers is a very promising method for reducing the spoilage fungi in the host.

When colonies or mycelial discs were irradiated, colony growth was inhibited. But recovery of colonies was observed even at the very high doses, probably due to a great number of cells present in a colony. Of all media tested, recovered colonies did grow again after a temporary halt of growth by the radiation. The duration of inhibition was increased when radiation dose was increased. Delay period was also dependent upon the nutritional condition of the media on which colonies were grown.

Irradiated CA retarded the initial growth of the fungus. Some of the components produced in the medium by radiation may inhibit the fungus. Chemical changes caused by radiation treatment in the fruits should be considered as they may influence the fungal growth.

**Changes in the Fungus Induced by the Irradiation**

Gamma radiation treatment of the conidia resulted in death of a high percentage of the fungus. Abnormal colonies and stable mutants with white spores or light green spores were found among the survivors. The
radio-resistance of their conidia was not higher than that of normal strain.

Cultures of *P. expansum* demonstrated strong polymethylgalacturonase and plant tissue maceration enzyme activities. The culture grown from irradiated conidia produced more of these enzymes than did the control conidia after 3 days of post-irradiation incubation despite lower mycelial growth. Evidently enzyme production was stimulated by irradiation. Some substances in the cell induced by irradiation may be responsible for this stimulation. Since the pectolytic enzymes are considered to be the main means of this pathogenic fungus to invade plants by softening their tissues, this stimulation could cause practical handicaps when radiation is applied to control post-harvest diseases. A thorough study of this kind of stimulation, both *in vitro* and *in vivo*, is necessary.

The nucleic acid content of the mycelium grown from the irradiated conidia were related to the growth stage of the fungus but not directly influenced by the irradiation. The growth of the fungus was slowed by irradiation of conidia and thus indirectly affected the nucleic acids content.

When thymine-2-C\textsuperscript{14} was used in broth medium, radioactivity was found both in the DNA and RNA fractions of the mycelium instead of being directly incorporated into DNA. No radiation induced nucleic acid degradation was observed in fungus during the 8 hours of post-irradiation incubation, either by chemical or isotopic analysis.
General Conclusions

In general, it was found that sensitivity of *P. expansum* to gamma radiation was influenced by such factors as nutritional condition of the media, age of spores, delayed plating, heat treatment, chemical sensitizers, and pre-irradiation of the medium. If the condition is properly selected, this fungus could be effectively inactivated by low doses of gamma radiation and thus retain the quality of fresh fruit and reduce the irradiation cost. Since considerable numbers of irradiated conidia recovered from injuries under conditions which slowed down or delayed the germination, the conditions for prevention of recovery should be studied more extensively.

Abnormal colonies and mutants were induced by the radiation. Some mutants were no more radiation resistant than the normal strain, but there is still a possibility that resistant mutants could be induced. Pectolytic enzyme production of the fungus was stimulated by the irradiation. Since the enzyme is an important agent in breaking down the host cell wall barrier, pathogenicity of the fungus is expected to be increased. The degradation of nucleic acids in irradiated mycelia was not detected by the analysis conducted; the changes may be too small to be measured.
LITERATURE CITED


Table 8. The formulae of the culture media

<table>
<thead>
<tr>
<th>Ingredients per liter</th>
<th>Czapek Solution Agar (CA)</th>
<th>Czapek Dox Broth (CDB)</th>
<th>Nutrient Agar (NA)</th>
<th>Potato Dextrose Agar (PDA)</th>
<th>Sabouraud Dextrose Agar (SDA)</th>
<th>Sabouraud Maltose Agar (SMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30 g</td>
<td>Sucrose</td>
<td>30 g</td>
<td>Potatoes, Infusion from</td>
<td>Neopeptone</td>
<td>Neopeptone</td>
</tr>
<tr>
<td>Sodium Nitrate</td>
<td>2 g</td>
<td>Sodium Nitrate</td>
<td>3 g</td>
<td>200 g</td>
<td>Dextrose</td>
<td>10 g</td>
</tr>
<tr>
<td>Dipotassium Phosphate</td>
<td>1 g</td>
<td>Dipotassium Phosphate</td>
<td>1 g</td>
<td>Dextrose</td>
<td>Maltose</td>
<td>40 g</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
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<td>Magnesium Sulfate</td>
<td>0.5 g</td>
<td>Agar</td>
<td>Agar</td>
<td>15 g</td>
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<tr>
<td>Potassium Chloride</td>
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<td>Potassium Chloride</td>
<td>0.5 g</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>0.01 g</td>
<td>Ferrous Sulfate</td>
<td>0.01 g</td>
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<td>Agar</td>
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<td>Neopeptone</td>
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<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>40 g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Agar</td>
<td>15 g</td>
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Table 9. Analysis of variance and means for colony diameter of *P. expansum* grown on the irradiated apple juice agar after 5 days of incubation

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-test value</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
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<td></td>
<td></td>
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<td>Treatments</td>
<td>2</td>
<td>0.0152</td>
<td>0.00760</td>
<td>0.432</td>
</tr>
<tr>
<td>Experimental error</td>
<td>21</td>
<td>0.3694</td>
<td>0.01759</td>
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</tbody>
</table>

LSD .05                    | 0.138              |

Table 10. Analysis of variance and means for colony diameter of *P. expansum* grown on the irradiated Czapek solution agar after 5 days of incubation

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F-test value</th>
</tr>
</thead>
<tbody>
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<tr>
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<tr>
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</table>

LSD .05                    | 0.102              |

LSD .01                    | 0.139              |

**Significant at 1 percent level**
Table 11. Analysis of variance and means for colony diameter of \textit{P. expansum} grown on the irradiated potato dextrose agar after 5 days of incubation

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>Mean squares</th>
<th>F-test value</th>
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<td><strong>LSD .05</strong></td>
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Table 12. Analysis of variance and means for colony diameter of \textit{P. expansum} grown on irradiated Sabouraud dextrose agar after 5 days of incubation

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>Mean squares</th>
<th>F-test value</th>
</tr>
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<tr>
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Table 13. Analysis of variance and means for dry weight of *P. expansum* grown in the irradiated Czapek Dox broth

<table>
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<th>Source of variation</th>
<th>Degrees of freedom</th>
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<tr>
<td>Replications</td>
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<td>4,432.32</td>
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<td>Treatments</td>
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<tr>
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LSD .05 15.94

Table 14. Analysis of variance and means for dry weight of *P. expansum* grown in the irradiated potato dextrose broth

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<th>Source of variation</th>
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<td>Sampling error</td>
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<td>123.51</td>
<td>5.146</td>
<td></td>
</tr>
</tbody>
</table>

LSD .05 3.55
VITA

Tsong-Wen Chou

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Effects of Gamma Radiation in Concurrence with Certain Environmental Conditions on Lethal and Physio-chemical Responses of Penicillium Expansum L.

Major Field: Food Science and Technology

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