POSTHARVEST INSTITUTE FOR PERISHABLES

MONOCLONAL ANTIBODY PRODUCTION AGAINST COLLETOTRICHUM GLOEOSPORIOIDES PENZ. A POTENTIAL TOOL TO DIAGNOSE ANTHRACNOSE IN MANGOES

by

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MONOCLONAL ANTIBODY PRODUCTION AGAINST *COLLETOTRICHUM GLOEOSPORIOIDES* PENZ. A POTENTIAL TOOL TO DIAGNOSE ANTHRACNOSE IN MANGOS

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CARMEN CALMA PATERNO

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MONOCLONAL ANTIBODY PRODUCTION AGAINST *COLLETOTRICHUM GLOEOSPORIOIDES* PENZ. A POTENTIAL TOOL TO DIAGNOSE ANTHRACNOSE IN MANGOS

ABSTRACT

by Carmen Calma Paterno

University of Idaho, 1986

Production of monoclonal antibodies against *Colletotrichum gloeosporioides* Penz. to detect anthracnose infection in mangos was demonstrated. Whole organism antigen induced a higher immune response than either mycelial or conidial antigens. Two isolates of *C. gloeosporioides* Penz. ATCC 32097 and ATCC 36036 were used as antigens. Immunizations of Balb/c mice were administered on day 1 and booster injections were administered on day 33. Fusion of immune spleen cells with myeloma cells P3X63Ag8.6543 was performed on day 36. A total of 11 hybridomas was obtained from two independent fusions. Two of the clones became non-producers. The cell lines produced IgM except for one which was a mixed culture of an IgM and IgG1. Analysis of the antigens showed ATCC 32097 to contain more protein than ATCC 36036. In addition, the antigenic determinant of ATCC 36036 was presumptively shown to be a carbohydrate. Specificity testing of the clones included two homologous and five heterologous antigens. Two clones were found to be specific to the homologous antigens.

INTRODUCTION

Anthracnose is a fungal disease that is widespread in tropical and subtropical fruits and vegetables. In mangos, the disease is caused by Colletotrichum gloeosporioides Penz., and is considered a primary factor in postharvest loss of the commodity.

The fungus attacks most of the aerial parts of the plant except the bark of the main trunk. On various parts of the plant, it causes leaf spot, leaf blight, peduncle blight, blossom blight, fruit stain, and premature fruit abortion or combinations of these.

Assurance of premium quality mangos for export is uncertain because of the manner in which the disease develops. It has been established that the organism causes "latent infections" in the fruit. In this type of infection, the causal agent penetrates early in the fruit developing stage and the disease becomes visible in the ripening stage. Since mangos are shipped at green mature stage, the possibility of packing unblemished but diseased fruits is always present.

Prevention of postharvest decay requires an integrated approach that includes field and packinghouse treatments. Timely fungicidal sprays can prevent infection of the plant parts in the field and conidia on the fruit surfaces can be washed off. Nonetheless, even with these combined measures, complete anthracnose control is difficult.

A tool to forecast decay potential of a given lot would be extremely useful. Forecasting would allow reduction of produce loss, production and shipping costs, and enhance market reputation.

This study investigated the use of hybridoma technology in the detection of anthracnose and describes production of monoclonal antibodies to C. gloeosporioides. The preliminary assay system developed and the results obtained are presented to show the specificity of the clones generated.

LITERATURE REVIEW

Mango production and losses

The mango is one of the most important commercial fruit crops of the world. Its excellent flavor, color and nutritional value have increased its popularity and market.

In 1981 the Food and Agricultural Organization (FAO) tabulated the mango production of the major producing countries:

Major Producers of Mangos

Country	1969-71	1978	1979	1980
India	8,300	9,000	9,300	9,500
Brazil	668	670	680	690
Mexico	298	541	566	610
Pakistan	485	561	538	560
Philippines	143	335	359	330
Haiti	257	310	318	326
World	12,276	13,751	14,067	14,342

Note: Figures are in thousand metric tons (FAO,1981).

In the trade process of fresh produce, decay by microorganisms is possibly the greatest cause of loss (Coursey, 1971). Decay is usually initiated by one pathogen and followed by a broad spectrum of biodegraders. In 1960, Singh reported that microbial decay of mangos ranged from 20 to 33% in exported commodity.

One form of microbial decay is anthracnose caused by C. gloeosporioides. Anthracnose is a plant disease characterized by sunken, necrosed limited lesions (Ainsworth, 1961). Nolla (1926) described symptoms on citrus, mango, and avocado as sunken spots initially ochraceous-orange in color turning to cinnamon-rufous to black as the disease progresses. Members of the Colletotrichum genus are known to cause this disease as well as stem rots, leaf spots, fruit rots, and "latent infections".

Anthracnose causes economic losses in a wide range of temperate and tropical crops. Postharvest reports quantitating losses of mangos due to anthracnose is minimal but in 1914 McMurran reported C. gloeosporioides to be the chief cause of mango crop loss in Florida. An economic report by Clarendon (1981) outlined in Table 1, pinpoints anthracnose as the major cause of the loss in Dominica:

TABLE 1. Postharvest Loss of Mangos due to Anthracnose in Dominica.

Year	Production (Tons)	% Loss	Value of Loss ($)
1978	235	40	12,633
1979	94	30	6,737
1980	90	30	6,451

When Mendoza (1980) ranked the causes of loss of Philippine mangos and he ranked anthracnose as the primary factor. Anthracnose-caused diseases resulted in a loss of 15% and other losses were due to stem-end rot (10%), immaturity (5%) and overripening (5%).

Control of mango anthracnose disease would benefit the local and foreign markets. To design an efficient control scheme, the capability to measure losses must be available. Kuchler et al. (1984) analyzed the economic consequences of a plant disease which spreads throughout the United States. The reduced yield initially will increase the price and be a benefit to the farmer but overall loss to the economy will be devastating. International effort to prevent disease outbreaks is also necessary. Crops to be introduced to other places must be screened for disease resistance in the new habitat (Lenne et al., 1984[b]).

Colletotrichum: latent infection

Classification of the organism according to Alexopoulos (1979) is as follows:

Superkingdom*: Eukaryonta

Kingdom: Myceteae

Division: Amastigomycota

Subdivision: Deuteromycotina

Class: Deuteromycetes

Order: Melanconiales

Family: Melanconiaceae

Genus: Colletotrichum

Species: gloeosporioides

(* in accordance with Wittaker & Margulis, 1978).

The organism is the anamorph or imperfect stage of Glomerella cingulata (Stonem.) Spauld. & Schrenk. Key to the classification of Melanconiales is conidia on acervuli which are cushion-like masses of hyphae. Characteristics of Colletotrichum are as follows: spores cylindrical, ellipsoidal or falcate, borne in succession on firm, basally often brownish, septate conidiophores (Ainsworth, 1961). After germination, C. gloeosporioides often forms appressoria, acervuli, and setae. Setae are stiff bristles around the acervulus.

A recent study of several Colletotrichum species by Lenne, Sonoda and Parbery (1984) showed strong evidence that conidium production by setae is a character of the genus.

Lenne (1978) observed the trait with isolates from Australia and Florida. This finding is also supported by reports of the species in Asia (Ikata, 1936; Ling and Liu, 1944; Van Hoof, 1950), and the United States (Sonoda et al., 1974). This character is therefore widespread. In Lenne's study, conidia produced from setal apices and conidiophores were identical. This characteristic is important to the transmission of the disease because conidia produced by setae are easily dislodged and dispersed (Lenne et al., 1984[a]; Nicholson and Moraes, 1980).

The causal organism was first studied and described by Penzig in 1882. It was not until the 1920's that mango anthracnose was differentiated from mango blight also caused by C. gloeosporioides (Nolla, 1926; Bessey, 1935). Researchers then investigated the infection process of the organism.

Infection is the act of penetration into a host tissue by a pathogenic microorganism. It has been observed that Colletotrichum members continued to exhibit delayed growth and penetration of the infection hypha after the initial infection (Baker, 1938; Chakravarty, 1957; Verhoeff, 1974). This was termed "latent infection" by Simmonds (1941) and defined to be the fungus' state of survival by initial penetration as contrasted to survival in the free state as a spore. The latency period is a dormant parasitic relationship which changes into an active one during ripening (Prusky et al.,

1984; Binyamini and Schiffmann-Nadel,1972).

There are several hypotheses on the physiological basis of change from a latent or dormant to active condition (Simmonds, 1941 and Verhoeff, 1974). There are three main points that may be the basis of this radical change: 1) presence of an antagonistic substance in the unripe fruit (Prusky et al., 1982); 2) significance of dependence on available nutrients (Prusky et al., 1984); and 3) activity of the pectinase enzyme (Verhoeff and Warren, 1972). The authors indicate that a specific substance prevents the activity of the organism. High concentration of tannin appears to have toxic effects (Verhoeff, 1974). No conclusive evidence was drawn concerning the significance of nutrients available to the fungus in dormancy (Verhoeff, 1974). Many arguments have been advanced regarding the influence of pectinase on growth and the presence of protopectin with similar and conflicting observations (Simmonds, 1963; Wood, 1967; Pladys et al., 1981; Adikaran et al., 1983). On this point, Verhoeff (1974) concludes that the specificity of each host-parasite combination be investigated separately. Temperature and ethylene production have also been considered to be factors in the initiation of activity change (Schiffman-Nadel and Michaeli, 1984; Ilag and Curtis, 1968).

Virulence or pathogenicity is the term used to describe the ability to infect (Beringer and Johnston, 1984; Dunkle, 1984). Dickman et al. (1982) attribute virulence to the

ability to produce cutinase enzyme (Purdy and Kolattukudy, 1973; Purdy and Kolattukudy, 1975; Baker and Bateman, 1978). Working with the papaya anthracnose organism, Dickman et al. proved that infection occurred with intact tissues. This finding eliminated the notion the C. gloeosporioides is exclusively a postharvest wound pathogen (Stanghellini and Aragaki, 1966). Quimio and Quimio (1975) also concluded that anthracnose spreads in the field. In the cross-inoculation studies with anthracnose organisms of mango, citrus and guava, the strains of pathogen were found to be host specific except for the guava organism. In a study by Dickman et al. (1982) host specificity is also apparent with cutinases. They found no cross-reactions between the cutinases isolated from the C. gloeosporioides and Fusarium solani (Mart) Appel & Wr. f. sp. pisi (F. R. Jones) Snyd & Hans..

Current methods of control

Control of the anthracnose disease currently involves both field and packinghouse operations. Basically, the treatments can be divided into chemical, hot water treatments, combinations of both and irradiation (Lakshiminarayana, 1980). The treatments generally reduce anthracnose but do not eradicate the fungus (Chang, 1975). Eckert (1983) enumerates the strategies and control agents for pathogenic microorganisms (Table 2).

Hot water treatment alone, and in combination with fungicides is employed and have become commercially obligatory practices in packinghouse operations for the export of mangos (Salunkhe and Desai, 1984). Reduced storage life, tissue injury and weight loss are the main disadvantages of the process compensated by better marketability (Lakshiminarayana, 1980). Subramanyam and Moorthy (1973) found that heat treatment with some fungicides caused a decrease in the sugar and an increase in the acid content of the fruit.

TABLE 2. Strategies for the Control of Pathogenic Microorganisms on Fresh Fruits and Vegetables (from Eckert,1983).

Strategy	Agents
1. Spray developing crop with fungicides to prevent pre-harvest infection	Protective fungicides, captan, maneb, etc.
2. Inactivate pathogens in postharvest environment	Broad spectrum sanitizing agents ---formaldehyde, hypochlorite, quaternary ammonium cpds.
3. Inactivate latent infections in harvested crops	Systemic fungicides, benomyl, thiabendazole
4. Eradicate spores in harvested crops	o-phenylphenate, sec-butylamine
5. Protect surface of product against infection through wounds created after the application of fungicide	Benomyl
6. Inhibit fungus sporulation and the spread of hyphae to adjacent fruits	Biphenyl, benomyl, SO_2, dicloran, metalaxyl

Thiabendazole and benomyl are generally employed for preharvest (Lakshiminarayana, 1980) and postharvest control of anthracnose (Salunkhe and Desai, 1984). Dipping the harvested fruit in a suspension of 1000 mg/l of thiabendazole or benomyl at 55 C prior to storage was found to be ineffective for the control of anthracnose (Jacobs et al., 1973 and Spalding and Reeder, 1972). Benomyl is widely used as a postharvest control for mangos. In a study on the effectiveness of benomyl on mango, Quimio and Quimio (1974) found 275 to 600 mg/l to be the lethal dose on the mycelium of Colletotrichum. However, Bondad (1974) reported that 200 to 500 mg/l resulted in fruit tissue injury. Bryan (1972) found positive and negative effects of benomyl on mango color and anthracnose.

Gamma radiation is also used to reduce postharvest spoilage. Thomas and Javane (1973) reported that irradiation of mangos at 200 kilorad (krad) reduced the ascorbic acid and increased the polyphenol oxidase activity which caused browning of the skin and pulp tissue. Working with lower doses of ionizing radiation to a maximum of 75 krad, Cuevas-Ruiz et al. (1972) maintained good fruit and prolonged storage life. Maxie et al. (1971) said that irradiation for perishable products with high moisture content is not feasible based on economic and health reasons.

Hybridoma technology

When a foreign antigenic substance is introduced into an

animal, a unique set of serum glycoproteins is produced which specifically reacts with the antigen (Unanue and Benacerraf, 1984). This discovery greatly enhanced the study of medicine and biology. Now serological techniques are routinely employed in medical technology, identification of pathogenic organisms, and the typing of blood for various purposes. However, two needs in utilizing the serum as a source of antibody were inadequately met. One was the need to reproduce the same consistent, standard, and specific reagents. The other was the need to be able to determine the quantitative composition of the product (Davis et al., 1982).

In 1975, Kohler and Milstein produced the first monoclonal antibodies based on previous works of other scientists (Table 3). Individual antibody-producing cells are hybridized with tissue culture-adapted plasma-tumor (myeloma) cells (Fox, 1979). The product is a cell (clone) which will proliferate producing one kind of antibody molecule. The trait to proliferate is inherited from the myeloma parent and the trait to produce the antibody comes from the immune cell parent.

The hybrid will produce homogenous antibodies directed against a single antigenic determinant. Monoclonal antibodies as contrasted to polyclonal (conventional) antibodies have the advantage of being pure and routinely obtainable from impure antigens. Monoclonals can be specific to a particular antigen-binding site in a complex mixture of antigens (Milstein, 1980). The capability to control the specificity

TABLE 3. Landmarks in the history of antibody research (from Goding, 1983).

1957	Clonal selection theory	Burnet
1958	One cell; one antibody Cell fusion by Sendai virus	Nossal and Lederberg Okada
1959	Elucidation of disulphide-bonded chain structure of antibodies	Edelman
1960	Discovery of spontaneous cell fusion	Barski
1962	Demonstration that Bence-Jones proteins are antibody light chains Induction of Plasmacytomas by mineral oil	Edelman and Gally Porter and Boyce
1962-3	Controlled proteolytic cleavage of IgG Identification of Fab and Fc; topographic relationship between light and heavy chains	Porter, Fleishman, Pain and Press
1964	Use of mutant cells and selective media to isolate hybrids	Littlefield
1965	Amino acid sequencing reveals that N terminal half of light chains is variable; C-terminal constant Postulate of two genes; one polypeptide	Hilschmann and Craig Dreyer and Bennett
1969	First complete amino acid sequence of an immunoglobulin; concept of domains	Edelmann and colleagues
1970	Hypervariable regions Growth of plasmacytomas in continuous culture	Wu and Kabat Horibata and Harris
1973	Fusion of mouse and rat myeloma cells with preservation of secretion of both immunoglobulins sets the stage for production of monoclonal antibodies	Cotton and Milstein
1975	Construction of hybridomas secreting antibody of predefined specificity	Kohler and Milstein

of hybrids makes the application of hybridoma technology diverse.

Applications

Monoclonal antibodies are used in the diagnosis, prevention and treatment of diseases in humans, animals and plants (Dixon et al., 1983) but it is difficult to categorize contributions of monoclonals into either the diagnostic, preventive or the treatment area. Halk and Franke (1983) found antigenic relationship using monoclonals among the viruses that cause apple mosaic, prunus necrotic ringspot, and tobacco streak. With the same tool, Diaco et al. (1983) found a common antigenic site shared by several isolates of the barley yellow dwarf virus. Differences of monoclonals to the barley yellow dwarf virus was studied by Hsu et al. (1984). Monoclonals specific to the potato leaf roll virus were produced and characterized by Martin and Stace-Smith (1983). All these studies aimed to diagnose, prevent, and eventually treat the plant disease by vaccine production.

Many plant diseases affect the production of crops for human and animal consumption as well as the development and maintenance of pathogen-free seeds and plants. To minimize the occurrence of widespread plant diseases, national and international programs identify major pathogens and prevent their spread in shipments by use of quarantine and certification procedures. Serology plays a vital role (Howard

et al., 1979) in these procedures but needs standardized immune reagents. Banowetz et al. (1984) tried unsuccessfully to develop a monoclonal against Tilletia caries (DC) Tul.(wheat bunt fungi) isolates found in the United States to certify quality of wheat shipments exported to other countries.

Production of monoclonal antibodies

The steps to the production of monoclonal antibodies are:

1. Immunization
2. Fusion and Primary Culture of Hybridomas
3. Cloning and Preservation of Hybrids
4. Production of Antibodies:
   a) In vitro
   b) In vivo

A variety of factors are involved in immunizing an animal to obtain a proper response. The antigen and the amount to use, the injection route, the schedule of immunization, and the choice of animal, all will affect the response and affinity of the antibody (Galfre and Milstein, 1981). For instance, antigens may be proteins (Higgins et al., 1980), carbohydrates (Hakomori and Kannagi, 1983) or lipopolysaccharides (Forbes et al., 1975) and immunogenicity is basically determined by the molecular weight of the antigen (Goding, 1983). Adjuvants are often used with the antigen to enhance an immune response (Herbert, 1973). Elaborate immunization schedules are not always necessary (Galfre and Milstein, 1981). The use of small quantities of immunogen is

more likely to induce a population of antibodies with high affinity and, consequently, sensitivity for the substance injected (Kohler and Milstein, 1975). Vaitukaitis (1981) carries this further by applying small doses of immunogen in multiple intradermal injections to stimulate maximum response. Purity of the antigen is not necessary but becomes important when impure materials give weaker specific responses (Kramer and Cremer, 1983) and when the screening method is not able to distinguish between antibodies to the specific component or to the impurities (Scharff, 1984). The amount of antibodies desired will be a factor in selection of the mouse or the rat as the animal to use in conjunction with the myeloma cell. Unless there are specific reasons, the myeloma should be of the same species as the immunized animal (Zola and Brooks, 1982).

The injected animal with the highest antibody level is chosen for the fusion step. The spleen of the animal is taken and cells from the spleen were fused with the myeloma cells. The Sendai virus was the fusing agent used for the first hybridomas. In 1976, Pontecorvo described the first polyethylene glycol (PEG) induced fusion of myeloma cells. PEG is now widely used because of higher fusion frequency, commercial availability, low price and ease of handling.

Three fusant products can arise from the fusion : 1) spleen-spleen, 2) myeloma-myeloma and 3) the desired

spleen-myeloma combination (Galfre and Milstein, 1981). The most common selection procedure is one devised by Littlefield (1964) known as the HAT (Hypoxanthine Aminopterin Thymidine) selection (Zola and Brooks, 1982). The method is based on the fact that when the main, de novo biosynthetic pathway is blocked by the folic acid antagonist aminopterin, an alternative "salvage" pathway to convert hypoxanthine or guanine to guanosine monophosphate is available via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Spleen cells contain the HGPRT while the myeloma cells do not and so the myeloma-myeloma fusants cannot survive. The spleen-myeloma combination will survive in the HAT medium by utilizing the alternative pathway. However, since it is not normal to use that pathway, the spleen-spleen combination eventually dies.

Cloning is the selection of positive hybrids which are derived from a single cell. This step is essential to determine that a culture contains only one type of cell and produces only one type of specific immunoglobulin. Cloning is performed by culturing single cells in individual wells and growing them into colonies. Cloning is best done as early as possible to prevent the overgrowth of negative hybrids or non-producers. This also prevents the danger of chromosome loss although it is not an insurance against it (Galfre et al., 1977).

Three ways of cloning are possible, namely: 1) by

limiting dilution (Galfre and Milstein, 1981); 2) by cloning in soft agar (Coffino et al., 1972); 3) by using the fluorescence-activated cell sorter (FACS) (Parks et al., 1979). Cloning by limiting dilution has the advantage of directly testing the supernatant fluids (Goding, 1983). It, however, does not guarantee monoclonality and therefore is recommended to be done twice (Goding, 1983; Galfre and Milstein, 1981). Cloning in soft agar or semisolid support is done with an underlayer of a gel matrix and soft agar overlay which contains the cells. Prior to testing antibody production, the cells must be plucked and recultured in liquid medium. Inability to test antibody production directly is the main disadvantage of soft agar cloning. This method is preferred by Galfre and Milstein (1981) and Iscove and Schreier (1979) because of better cloning efficiency. Cloning using the FACS involves light scatter characteristics to identify the healthy cells, mixed cells and the dead cells. The advantage of the FACS is the capacity to select cells of the desired specificity. Davis et al. (1982) developed a variation of the soft agar cloning method without an underlayer support. Hybridomas are selected and cloned in one step immediately after the fusion by plating the cells in semi-solid medium containing methylcellulose and the components of the HAT selection system. This single step technique eliminates routine recloning and allows the handling of large number of cells.

The step in the selection process which is most important

is the assay system. An appropriate assay system should be sensitive and easily performed. It should give the maximum amount of information in relation to the desired product of the fusion. Davis et al.(1982) described several assay systems. Cytotoxicity assays are done to test monoclonals that contain complement-fixing antibodies against cell surface antigens. Complement is added to wells that have been washed of unbound antigens and the lysis is examined under the microscope. Screening by immunofluorescence and flow cytometry (Loken and Stall, 1982) both use a fluorescent agent to detect binding. The antigen and the hybridoma supernatant fluid is combined at a low temperature and the fluorescein conjugated anti-immunoglobulin antibody is added to the mixture. Reaction indicated by the presence of the fluorescence is examined under the microscope or the FACS. The difference between the two methods is the use of laser beam for quantitation of antigen-antibody reaction in flow cytometry. The two processes can also be used together (Parks et al., 1979).

Radioimmunoassay is another screening system that was commonly used. The drawback of the assay is the use of radioisotope ^{125}I. In place of the radioisotope, Engvall and Perlmann (1972) replaced the reagent with a conjugated enzyme. Basically, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) are microplate techniques that involve a series of incubation and washing of antigen-treated

micro-well plates treated with hybridoma supernatant fluid, an anti-immunoglobulin and substrate to highlight the reaction. Reaction is measured using a spectrophotometer. The ELISA is simple, rapid, highly reproducible, economical, can handle large numbers for testing, and does not use radioactive reagents. Because of the advantages it offers, the assay has gained popularity and acceptance (Posner et al., 1982; Clark, 1981; Richardson and Warnock, 1984).

After the positive cell lines are detected, the next step is to produce the monoclonal antibody in quantity and quality. This can be done either in vitro or in vivo (Galfre and Milstein, 1981). In vitro production is done by tissue culture and is expensive because of the cost of serum and the amount of labor involved in the process. Galfre and Milstein (1981) prefer the tissue culture because the experimenter has control over the protein impurities that can possibly gain entry to the cultures. The in vivo method entails injecting the hybridomas intraperitoneally into the mice to mass produce the monoclonal antibodies in the ascitic fluid which is collected. The main advantage of in vivo production besides cost-saving is the high concentration of the serum obtained, which reportedly can reach up to 4 times that obtained in tissue culture (Galfre and Milstein, 1981).

No hybridoma work has been reported with C. gloeosporioides. The production of monoclonal antibodies to this fruit and vegetable pathogen could result in the

development of a simple, reliable serologic test for diagnostic uses in field and packinghouse operations.

MATERIALS AND METHODS

Stock cultures

Four cultures were obtained from the American Type Culture Collection (ATCC). Three were Colletotrichum gloeosporioides Penz. with the following accession nos.: 32097, 36036, and 38237. The other was C. gloeosporioides var. minor Simmonds ATCC 42374. Mango was the source of isolates 38237 and 42374. ATCC 36036 was isolated from grapefruit and 32097 from grapes by Quimio (ATCC Catalogue,1982).

Production and preparation of antigens

All isolates were grown on potato dextrose agar (PDA). Aliquots of 100 ml agar were poured into roux bottles and sterilized at 15 lbs pressure (121 C) for 15 min. Two ml of distilled water was added to the lyophilized cultures from ATCC. A loopful of this suspension was used to inoculate each bottle. Incubation was at 25 C for 3 weeks.

Spore, mycelia and whole organism preparations were produced as follows. Cultures were generally harvested by the methodology of Kwapinski (1965). A solution of 0.85% saline and 0.05% Tween 80 was heated to dissolve the Tween 80 and was used to wash the cultures off the agar. Fifty ml of solution was added to each bottle of culture. The growth was scraped thoroughly with a loop. For spore and mycelial harvests,

washings were filtered through layers of sterile cheese cloth. Mycelial residue was washed off the cloth and collected. Washings of the three antigen preparations and collected residues were centrifuged at 12,000 x g for 10 min. The supernatant fluid was discarded and the pellets were resuspended in distilled water. The process of centrifugation was repeated three times. The suspensions obtained were poured into sterile vials and were lyophilized. These lyophilized antigen preparations were used throughout the study.

Animals

Eight wk old New Zealand white female rabbits, weighing approximately 1.5 kg each, were obtained from Rabbits Galore, 1050 Vineland Dr., Clarkston, Washington. The rabbits were housed singly in stainless steel cages at the Small Animal Laboratory at the University of Idaho.

Female BALB/c mice were obtained from Laboratory Animal Resource Center, Washington State University, Pullman, Washington. The mice were housed singly in an Econofilter (Maryland Plastics, Inc., New York, N.Y.) covered cage.

All animals were allowed free access to food and water.

Induction of immune response

Rabbits were injected with either the conidia, mycelia, or the whole organism to determine the immunogenicity of the

substance.

Rabbits were injected with a total of 30 mg of the appropriate antigen emulsified in 2 ml of Freund's complete adjuvant on day 1. Fifteen mg of antigen in 1 ml saline was administered intramuscularly (I.M.) on day 21, 23, 25, and 30. On day 39, the animal was boosted I.M. with 1 ml of antigen without adjuvant.

The animals were bled via the marginal ear vein on days 21 and 39 to evaluate antibody activity. Animals were exsanguinated by cardiac puncture on day 46.

Freshly collected blood was incubated 25 C overnight and allowed to clot. It was then centrifuged at 400 x g for 5 min to separate the serum and the coagulated plasma fraction. The serum was aspirated from the tubes with a pasteur pipet.

Slide agglutination test was employed to determine the titer of rabbit antisera (Kwapinski, 1972). A two-fold serial dilution of the serum was made using Kolmer's saline solution (0.9%) as the diluent. After thorough mixing, equal amounts of antigen suspension and the appropriate antiserum dilution were mixed on a slide. Results observed were expressed on a scale of negative (no agglutination) to a four plus (100% agglutination). Also with the use of the slide agglutination test, cross-reactions of antigens and antisera were performed to select the cross-reactive antigen desired. The antigen that elicited the strongest response

was used for immunization of the mice for spleen cells for fusion.

Determination of protein in the antigen

Protein content of the antigens was determined by the Lowry method (Lowry et al., 1951) as follows. Three reagents were used in this test: reagent A was a 2% (w/v) Na_2CO_3 (Sodium carbonate, anhydrous) in 0.1 N NaOH; reagent B was a 0.5% $CuSO_4.5H_2O$ in 1% $Na_2C_4H_4O_6.2H_2O$ neutral (sodium tartrate-neutral) pH adjusted to greater than 6.0; reagent C was a mix of 50 ml of reagent A plus 1 ml of reagent B. Briefly, standard samples of 0.6 ml containing 5-100 ug of bovine serum albumin was prepared. Three ml of reagent C was added, mixed and let stand at 25 C for 15 min. Then 0.3 ml of 1 N Folin's ciocalten reagent was added and mixed. The solution was allowed to stand at 25 C for 35 min and the absorbance at 500 nm was determined.

The amount of protein was calculated using linear regression and the method of least squares.

Immunization and assay schedule of mice

Six wk old female BALB/c mice were injected intraperitoneally (I.P.) with 0.1 ml emulsion of equal parts whole organism antigen (15 mg/ml) and Freund's complete adjuvant. On day 33, a booster injection of 0.1 ml of

antigen preparation was given I.P. Blood samples from the tail were collected by cutting half an inch off the tail end. After incubation at 37 C for 1 h and allowing the blood samples to stand at room temperature overnight and centrifuging, the serum fraction was collected. Antibody activity was evaluated by slide agglutination test as previously described.

On day 36, the mouse with the highest titer was killed by suffocation with anhydrous ether (Mishell, et al., 1980). With a tuberculin syringe, blood was immediately collected by cardiac puncture and was discarded. The mouse was washed with 70% ethanol and pinned on the paraffin pan with its stomach facing up. Under aseptic conditions, the stomach skin of the mouse was lifted with sterile forceps and a crossing incision was made with sterile scissors. The skin was carefully pulled away from the incision being careful not to allow the scissors to touch the skin and then an incision into the peritoneal cavity just above the spleen was made. The spleen was removed from the fatty membranes and placed in a plate with RPMI 1640 (Roswell Park Memorial Institute) medium. The spleen was teased through a mesh screen to make a cell suspension for the fusion.

Fusion, hybridoma selection and cloning

A count of 3.4×10^7 spleen cells and 1.36×10^8 myeloma cells (supplied by Claudia Beck, U of I) were mixed

together in a graduated centrifuge tube. The suspension was mixed and then centrifuged at 400 x g for 10 min to form a tight pellet. After removal of the supernatant fluid, 1 ml of 50% polyethylene glycol (PEG) in RPMI 1640 was added slowly over a 1 min period, gently stirring the cell pellet with the tip of the pipette. The cell pellet was stirred for an additional minute. Using the same 1-ml pipette, 2 ml of serum-free RPMI was added slowly to the cells at a rate of 1 ml/min. Seven ml of serum-free medium was then added stirring continuously over 2-3 minutes. The suspension was then centrifuged at 400 x g for 10 min and the supernatant fluid was discarded.

The fusion pellet was resuspended in 15 ml of solution which contained 53.3% v/v fetal calf serum (FCS), 2.66% v/v hypoxanthine aminopterin thymidine (HAT) stock solution, 8 x 10^6 thymus cells/ml and 133 ug lipopolysaccharide (LPS)/ml in Dulbecco's modified Eagle's medium (DMEM). With a 10-cc syringe without needle, 25 ml of 2% w/v methlycellulose was added (Davis et al., 1982) to the solution. The suspension was mixed gently until a homogeneous mixture was obtained. The mixture was dispensed in 1-ml portions to small tissue culture dishes (35 x 10mm, Falcon 3001) using a 10 cc syringe equipped with a 16-gauge needle. These covered dishes together with an open dish of water were placed in 150 mm petri dishes (VWR, Seattle, WA 98188). The plates were incubated in a water-jacketed CO_2 incubator (Napco)

containing 7% CO_2 at 37 C.

Plates were examined daily for colonies. Visible colonies were transferred singly to wells of a 96-well tissue culture plate (Costar, Cambridge, MA, Cat. No. 3596) with sterile capillary pipets. A pasteur pipet was used to deliver 1 drop of medium to each well. Medium was RPMI 1640 with penicillin and streptomycin plus 15% FCS (defined, Hyclone, Logan, UT 84321). Replenishment of medium became more frequent as the concentration of the hybrids became more dense and the medium turned slightly yellow (Oi and Herzenberg, 1980).

Assays by ELISA

The enzyme-linked immunosorbent assay (ELISA) was utilized to screen for positive hybridomas, to evaluate titer of the ascitic fluid, to observe cross-reactions, and to determine the biochemical nature of the antigen.

The technique adapted was the microplate modification as described by Voller et al. (1974). The controls used in all of the screenings were the normal serum and the enzyme conjugated antibody controls. Polystyrene microtiter plates (Corning Glass Works, Corning, N.Y., Cat. No. 25860) were incubated at room temperature for 30 min with poly-l-lysine (Cat. No. P-1399, Sigma, St. Louis, MO 63178) at 10 µg/ml in 0.01M PBS (pH 7.4). The plates were rinsed once with deionized water. To each well 50 µl of 500 µg/ml of

lyophilized whole cell antigen in deionized water was added. The plates were dried overnight in a vacuum dessicator. The wells were washed once with PBS. To reduce non-specific binding, 100 µl of 0.1% bovine serum albumin (BSA) (Cat. No. 7888, Sigma, St. Louis, MO 63178) in PBS was added. After a 30-min incubation at 37 C, the wells were rinsed with PBS twice, and 100 µl of hybridoma supernatant fluid was added to each well. For the serum control wells, RPMI 1640 with 15% FCS was used instead of the hybridoma supernatant fluid. After 1 h incubation at 37 C, the plates were washed three times with PBS, twice with deionized water and 100 ul of specific anti-immunoglobulin conjugated to an enzyme (Horseradish peroxidase-conjugated goat anti-mouse IgG + IgM; Code 6443,Tago Inc., Burlingame, CA 94010) was added to appropriate wells. PBS was added to the conjugate controls.

After incubation at 37 C for 45 min, plates were washed three times with PBS and twice rinsed with deionized water and 100 µl of 0.08% 5-amino salicylic acid (pH 6.0) in deionized water plus 0.05% hydrogen peroxide (added just prior to use) was added to each well. The plates were incubated in the dark for 1 h at 37 C. The optical density of the wells was measured at 405 nm using an EIA reader (Bio-tek Inc., Burlington, VT, EL 307) zeroed with the conjugate controls. Hybridomas with a consistent absorbance greater than 0.25 in five assays were considered positive

and selected for further study.

Presence of enzyme activity in the antigen was checked by two simple microplate tests which eliminated the inclusion of the hybridoma supernatant fluid. The first test included the antigen, BSA and rinses plus the salicylate while the second involves only the antigen plus the salicylate or the indicator. Presence of reaction was measured at 405 nm using the EIA reader. The purpose of checking the presence of enzyme activity in the antigen was to ascertain whether the results obtained were true positives and not false positives caused by non-specific antigen-enzyme binding.

Nomenclature of hybridomas

The hybridomas were labeled as explained in the following example: $1Y_2B_3$

1= the number of the fusion using the antigen designated by the following letter.

Y= organism ; Y = C. gloeosporioides Penz. ATCC 36036
X = ATCC 32097.

2= the fusion plate number of the clone.

B= the row occupied by the clone.

3= the column occupied by the clone.

Determination of antibody isotype

Sub-isotyping was performed using a sub-isotyping kit from Hyclone Immunochemical Products (Cat. No. 55050-K,

Hyclone, Logan, UT 84321). The kit provided all reagents and the protocol which utilized a microplate double antibody detection system. The following was the procedure performed. One ml of the plate coating solution was diluted with 9 ml of distilled water for each plate to be coated. For 10 ml of the diluted plate coating solution, 100 µl of goat anti-mouse immunoglobulins was added and 100 µl of this mixture was put into each well of 96-well plates (Cat. No. 25860, Corning). After the plates were incubated 18 - 24 h at 4 C, the contents of the plates were washed off with PBS three times. Plates were patted dry on a clean towel and 50 µl of PBS plus 50 µl of the hybridoma supernatant fluid was added to the wells. The plates were incubated at 25 C for one hour. Contents of the plates were washed twice with water and then the plates were patted dry on a towel. Two drops of one of the following typing antisera rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA were added to a different well for each hybridoma tested and the plates were incubated at 25 C for 1 h. Antisera was washed off the plates with water twice and 100 ul of peroxidase conjugate diluted to 1:4000 with PBS was added to each well. The plates were incubated at 25 C for 1 h and again washed with water twice. After patting dry on a towel, 100 µl of substrate-chromaphore was added to each well. The substrate reagent was a concentrate of citrate buffer containing 1% urea peroxide. Plates were incubated at 25 C for 20 min and wells were observed for the development of

a yellow, amber color which indicated presence of the specific antibody type tested for. Negative wells were pale yellow.

Ascitic fluid production

To increase the production of antibodies, the hybridomas were injected I.P. into female, 8-wk old BALB/c mice. Prior to injection of 10^6 - 10^7 cells, the mice were primed I.P. with 0.5 ml pristane (tetramethylpentadecane, Aldrich Chem. Co., Milwaukee, WI 53201) to increase the antibody production response. After a week, cells rinsed free of FCS were injected into the mice. When tumors were developed after 2 to 3 wk , the mice were suffocated in a beaker with anhydrous ether. To prevent possible contamination, blood was removed with a tuberculin syringe by cardiac puncture. With aseptic forceps and scissors, the skin was cut and peeled off enough to expose the abdomen. A 3-cc syringe with a 25-gauge needle was pricked to the side of the abdomen with the bevel of the needle facing up to prevent the fluid from spilling out. Slowly the ascitic fluid was suctioned out with the needle by moving the needle around inside the abdomen. The fluid was emptied into a graduated centrifuge tube, which was allowed to stand undisturbed for 10 min while the pristane formed the upper layer. The pristane layer was aspirated with a capillary pipet and discarded. The fluid was centrifuged at 400 x g for 10 min and the ascitic fluid was put into eppendorf tubes and frozen at -20 C until the titer was

determined by ELISA.

Determination of the biochemical nature of the antigen

To determine the biochemical nature of the antigen, the Colletotrichum antigens were investigated according to steps described by Stephens et al. (1982). Two hybridomas of each isolate were selected for the study. Antigen preparations of 300 µg/ml in deionized water were subjected to three treatments: (1) heat, (2) pronase, and (3) periodate oxidation. Antigen was heated in a water bath for 30 min at 80 C and was added to a microtiter plate. Proteinase K (Cat. No. P 0390, Sigma, St. Louis, MO 63178) was substituted for pronase. To plates with organisms, 50 µl of 100 µg/ml proteinase K was added and incubated at 37 C for 2 h. Periodate treatment was by the addition of 50 µl of 0.05M sodium m-periodate (Sigma, Cat. No. S 1878) to organisms in the well. Oxidation was performed at 4 C for 24 h. All plates were washed with PBS and assayed by ELISA.

Determination of specificity of cell lines

The fungi used for this study are listed in Table 4. All cultures were grown in the prescribed media (Table 4) and incubated at 25 C for 5 days. The cells were harvested and washed with 0.01M PBS (pH 7.4). To coat plates, cells were resuspended in PBS and adjusted to an absorbance of 0.5 at 500 nm. After drying overnight in a vacuum dessicator, ELISA was

performed with the supernatant fluid of all the selected hybridomas. Controls included were the enzyme-conjugate control, serum, and the antigen control. This test was performed to determine the specificity of the monoclonals.

Absorbances at 405 nm were read and evaluated using the ANOVA and Tukey's multiple comparison of means (Steel et al., 1960; Christensen, 1977).

TABLE 4. Fungi employed in Cross-Reaction Experiments.

Fungi	Media	Source
1. Alternaria alternata	Potato Dextrose Agar	ATCC 3409
2. Aspergillus niger	Malt Extract Agar	University of Idaho reference culture (Dr. A.J. Lingg)
3. C. gloeosporioides	Potato Dextrose Agar	ATCC 38237
4. C. gloeosporioides isolated from papaya	Potato Dextrose Agar	Wash. State Univ. reference culture (Dr. M. Dickman)
5. Fusarium moniliforme	Malt Extract Agar	University of Idaho reference culture (Dr. D. Crawford)
6. Phytophthora cactorum	Cornmeal Agar	University of Idaho reference culture (Dr. A. Helton)
7. Phytophthora megasperma	Cornmeal Agar	University of Idaho reference culture (Dr. A. Helton)

RESULTS

Stock cultures

The growth characteristics of four C. gloeosporioides isolates were similar except for pigmentation and sporulation. Pigmentation varied from the color orange (ATCC 32097), to brown (ATCC 38237), grey (ATCC 42374) and black (ATCC 36036). Mycelia of all the isolates were white. By microscopic observation, spores of all isolates were ellipsoidal. Isolate 42374 did not sporulate as well as the rest of the cultures. Consequently it was not used in the study.

Induction of immune response

A summary of the immunogenicity of the C. gloeosporioides antigens as detected by slide agglutination is presented in Table 5. None of the mycelial antigens induced a response. Conidia elicited a reaction up to a titer of 1:80 for isolate 36036 and 1:20 for both isolates 32097 and 38237. The whole organism antigens induced responses only in two isolates 32097 and 36036 with titers of 1:80 and 1:40, respectively. Cross-reactions demonstrated the ability of the whole organism antigen to detect the spore antisera. The whole organism antigens of isolates 32097 and 36036 were selected for mice immunization.

Determination of protein in the antigen

Using the linear regression and method of least squares, the protein content of whole organism antigens were found to be 184.6 µg/ml and 96.9 µg/ml for 32097 and 36036, respectively. Calculations and the plot of points on the standard curve are presented in Appendix 1.

Fusion

Fusions with cells from mice injected with ATCC 32097 failed twice. An inadequate number of spleen cells was suspected to be the reason for failure so two mice were utilized for the third fusion which produced 480 hybridomas. The titers of the two mice before fusion were 1:80 and 1:100. The titer of the mouse injected with ATCC 36036 was 1:120.

One hundred and ten hybridomas were obtained from the ATCC 36036 fusion and 480 were obtained from the fusion with

TABLE 5. Titers of sera from rabbits to C. gloeosporioides whole organism, spore, and mycelial antigens as detected by slide agglutination.

Antigen	Isolate Number		
	32097	36036	38237
Mycelia	0	0	0
Spore	20*	80	20
Whole organism	80	40	0

* Antibody level expressed as the reciprocal of the highest dilution of serum giving agglutination.

ATCC 32097. Recovery of positive hybridomas was 6% for the ATCC 36036 fusion and 0.8% for the fusion with ATCC 32097. These recovery percentages may reflect the antigenicity of the isolates. Table 6 shows the absorbance of the protein or antibody of the selected positive hybridomas.

TABLE 6. Absorbance at 405 nm of antibodies produced by C. gloeosporioides hybridomas.

Hybridomas	$A_{405\ nm}$
$3X_2E_7$*	0.381***
$3X_4C_5$	0.368
$3X_7E_3$	0.613
$3X_8F_8$	0.580
$1Y_1B_5$**	0.264
$1Y_3D_7$	0.346
$1Y_3D_8$	0.588
$1Y_3D_{10}$	0.543
$1Y_2B_8$	0.334
$1Y_3D_6$	0.467
$1Y_3D_9$	0.311

* X = ATCC 32097
**Y = ATCC 36036
*** = mean of 5 replicates

Determination of antibody isotype

The immunoglobulin sub-isotype of the antibody produced by all the hybridomas was IgM. Cell line $3X_8F_8$ was found to produce IgM and also had a slight positive reaction indicating presence of IgG_1. Cell lines $1Y_3D_7$ and $1Y_3D_8$ were found to be negative variants.

Ascitic Fluid Production

Ascitic fluid was produced from cell lines $3X_2E_7$, $3X_7E_3$, and $1Y_3D_{10}$. The titers of the ascitic fluids from the three lines were 1: 2600, 1:3000 and 1:3000, respectively. The amounts of fluid obtained ranged from 3 to 7 ml.

Biochemical nature of the antigen

Evaluation of the biochemical nature of antigenic determinants in two hybridomas of each isolate is presented in Table 7. Absorbance at 405 nm show that ATCC 36036 was neither destroyed by heat nor the proteinase treatment but was denatured by periodate indicating a carbohydrate nature. ATCC 32097 appeared to be a protein. It was sensitive to heat and proteinase but was not destroyed by the periodate treatment.

TABLE 7. Absorbance at 405 nm of Elisa between treated antigen and hybridomas.

Antigen Treatment	Hybridomas			
	$1Y_2B_8$	$1Y_3D_{10}$	$3X_2E_7$	$3X_7E_3$
Heat	0.425	0.825	0.054	0.073
Pronase	0.973	0.532	0.062	0.087
Periodate	0.103	0.126	0.316	0.469

* Y = ATCC 36036
**X = ATCC 32097

Determination of specificity of cell lines

Means of $A_{405\ nm}$ values for the cross-reactions are presented in Table 8. The analysis by ANOVA and Tukey's multiple comparison of means show which cell lines gave

TABLE 8. $A_{405\ nm}$ means of reactions between hybridomas and other fungi analysed with ANOVA and Tukey's multiple comparison of means at 5% level of significance.

Hybridomas	Fungi						
	1*	2	3	4	5	6	7
$3X_2E_7$	0.061	0.028	0.075	0.039	0.060	0.008	0.008
$3X_4C_5$	0.494_a	0.187_a	0.064	0.037	0.063	0.073	0.002
$3X_7E_3$	0.005	0.013	0.121	0.071	0.110	0.117	0.002
$3X_8F_8$	0.557_a	0.143_a	0.071	0.048	0.071	0.052	0.004
$1Y_3D_{10}$	0.039	0.079	0.761_a	0.596_a	0.678_a	0.575_a	0.006
$1Y_2B_8$	0.049	0.014	0.071	0.092	0.059	0.155	0.006
$1Y_3D_9$	0.095	0.014	0.082	0.023	0.072	0.048	0.004
Control	0.078	0.013	0.081	0.093	0.074	0.086	0.041

a= significantly different
*1= C. gloeosporioides ATCC 38237
2= C. gloeosporioides isolated from papaya
3= Aspergillus niger
4= Phytophthora cactorum
5= Phytophthora megasperma
6= Fusarium moniliforme
7= Alternaria alternata

significant reactions to the organisms at a 5% level of significance. From the computations (Appendix 2), the calculated control value is 0.1. If the difference of the means and this control value is greater than 0.1, then the reading is significantly different. Two cell lines observed to have positive reactions with C. gloeosporioides were $3X_4C_5$, $3X_8F_8$ and $1Y_3D_{10}$ was strongly cross-reactive with heterologous fungi .

DISCUSSION

Specific monoclonal antibodies can be produced against Colletotrichum gloeosporioides nevertheless, the ability of the organism to elicit a high immune response requires more investigation. Although responses were elicited with conidial and whole organism antigen, the titers were not high. Also, IgG was not induced other than in one hybridoma which was a mixed culture, cell line $3X_8F_8$. This culture will require cloning to separate the IgG and IgM producers.

A possible reason for low immune response was the choice of antigen material. Whole organism, as supported by the findings, was probably the best antigen or source of antigen material since it also detected conidia.

Purified protein of the isolates may be strongly immunogenic as proteins are believed to have such action (Kwapinski, 1972). However, specific characteristics for strong immunogenicity is poorly understood. Not all proteins are immunogenic (Banowetz et al., 1984) and purity is not the only factor of antigenicity (Unanue and Benacerraf, 1984). Also, there are adjuvants to increase antigenicity of a substance.

Results of the protein determination and biochemical nature of the antigens appear to be consistent with each other. Both tests evaluated the nature of the antigens. The Lowry method quantitatively determined the aggregate protein

in the antigens while the physicochemical test gave an idea of the nature of the antigenic determinant in each isolate. Isolate 32097 had 184.6 µg/ml protein and the antigen from this isolate was shown to be protein by the biochemical test. Isolate 36036 was showr to have only 96.9 µg/ml protein and the antigen was a carbohydrate according to the periodate test.

The antibody isotype of all cell lines was IgM except for one X hybridoma which was thought to be a mixed culture containing both IgM and IgG1 producing cells. IgG antibodies have higher affinity than IgM which relatively have low affinity that do not usually increase with time (Gearhart et al., 1981). Isolate X (32097) generated four hybridomas namely: $3X_2E_7$, $3X_4C_5$, $3X_7E_3$ and the mixed culture $3X_8F_8$. The specificity study showed that clones $3X_4C_5$ and $3X_8F_8$ reacted with both of the C. gloeosporioides; isolate ATCC 38237 and that isolated from papaya. The detection of homologous antigens was the desired specificity. Among the three Y (36036) hybridomas tested, none reacted with the homologous antigens. Hybridoma $1Y_3D_{10}$ was strongly cross-reactive with the heterologous antigens.

Clone $3X_4C_5$ and $3X_8F_8$ may recognize an antigenic determinant shared by most C. gloeosporioides. The $3X_4C_5$ and $3X_8F_8$ monoclonal antibodies may be useful reagents for a diagnostic kit for anthracnose infection. However, three homologous antigens are not a sufficient number among the known and existing isolates of C. gloeosporioides .

Another possible reason for the low titer was the use of the agglutination test which is not a sensitive test. It is a fast, easy and reliable test and has been used successfully for serological studies on plant pathogens (Romeiro, 1983).

It is assumed in this study that reactions observed in the laboratory will be observed under field test conditions. Actual detection of C. gloeosporioides infection may involve adverse conditions which could disrupt the affinity of the clones.

This study increases the possibility of a diagnostic kit to detect anthracnose infection. It would be a service to mango growers, traders and consumers. The potential use of the clone to detect anthracnose would have a significant effect on mango marketing.

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APPENDIX 1. Protein content of antigen using linear regression and method of least squares.

Concentrations (µg/ml) and mean absorbance values at 500_{nm} of three replicates dilutions of the bovine serum albumin (S-standard) and the antigens:

Dilutions	Protein levels	Mean $A_{500\ nm}$
S_1	83	0.047
S_2	170	0.162
S_3	333	0.406
S_4	500	0.582
X		0.186
Y		0.072

Using linear regression and method of least squares, the equation $Y = 0.0013\ X - 0.54$ was obtained. Substituting the absorbance values in the equation, the following answers were obtained.

For sple X:

$0.186 = 0.0013X - 0.054$

$\frac{0.2400}{0.0013} = \frac{0.0013X}{0.0013}$

184.6 µg/ml = X

For sple Y:

$0.072 = 0.0013Y - 0.054$

$\frac{0.126}{0.0013} = \frac{0.0013Y}{0.0013}$

96.9 µg/ml = Y

The graph of absorbance and protein concentration is shown in Fig. 1.

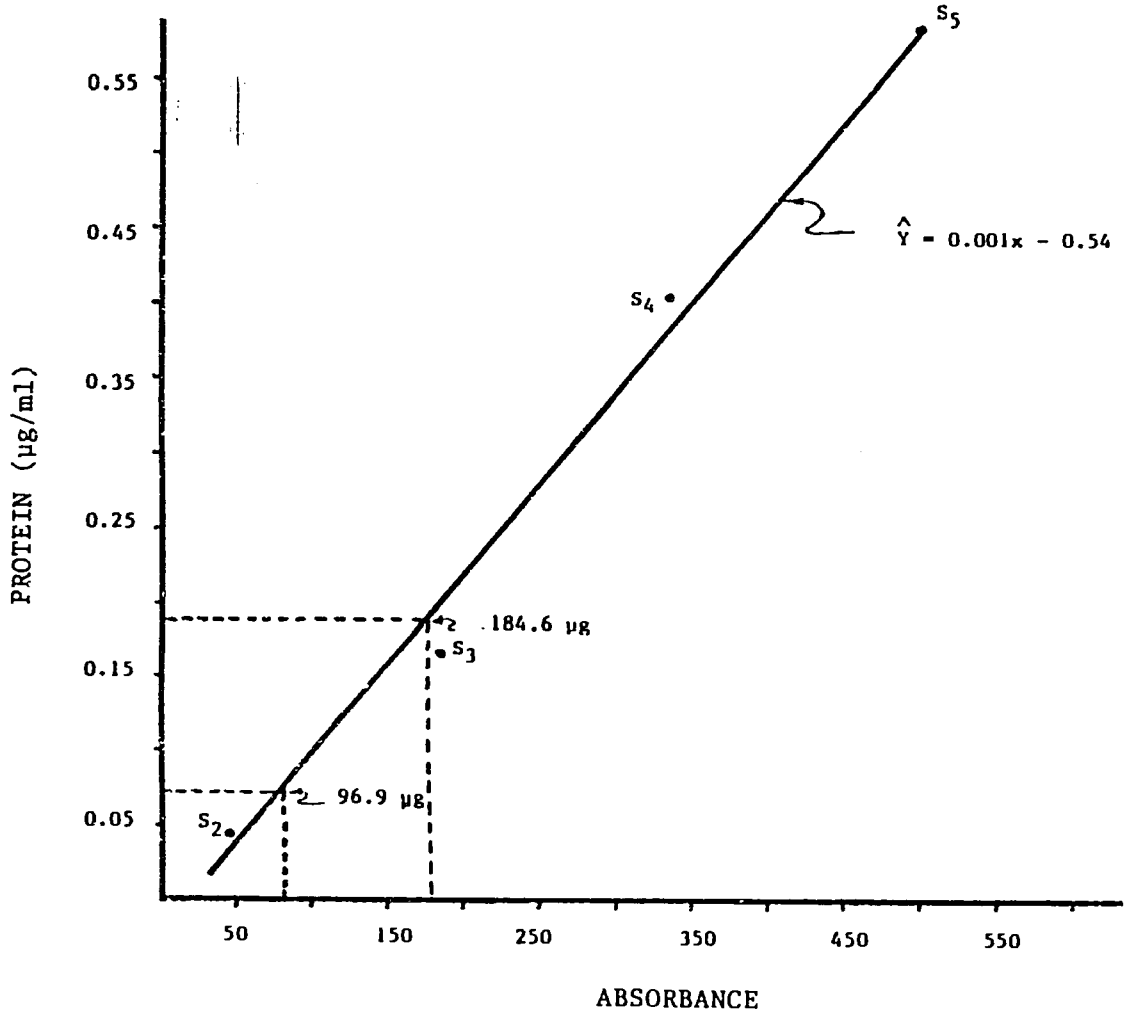

FIG. 1. Graph of Absorbance vs. Protein Concentration Using the Lowry Protein Method

APPENDIX 2. ANOVA and Tukey's Multiple Comparison of Means.

1. The ANOVA table using 7 x 8 factorial experiment.

Source	$F_{computed}$		$F_{tabulated}$	Conclusion
Organisms	2.1716	<	2.34	not significantly different
Cell Lines	3.25	>	2.25	significantly different

2. Multiple Comparison of Means using Tukey's Procedure.

a) Index to evaluate means among organisms:

$$|Y_i - Y_j| \geq 0.23311$$

Results showed no significant difference among the organisms.

b) Index to evaluate means among hybridomas:

$$|Y_i - Y_j| \geq 0.4007$$

Results showed that cell line $1Y_3D_{10}$ is significantly different from the rest of the cell lines.

c) Index to evaluate means of individual reactions:

$$|Y_i - Y_j| \geq 0.107575$$

Results showed that hybridomas $3X_4C_5$, $3X_8F_8$, and $1Y_3D_{10}$ are significantly different.