ANNUAL REPORT TO

THE UNITED STATES GOLF ASSOCIATION

GREEN SECTION

Project: MONOCLONAL ANTIBODIES FOR RAPID DIAGNOSIS OF PATCH AND NECROTIC RING SPOT DISEASES OF TURFGRASS

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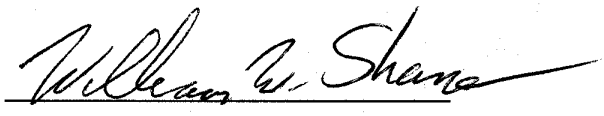

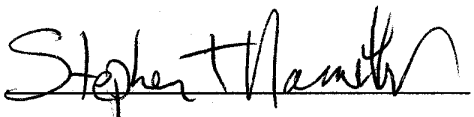

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EXECUTIVE SUMMARY

Summer patch and necrotic ring spot diseases of Kentucky blue grass, annual blue grass, and other turf grasses are extremely difficult to diagnose with traditional techniques. Research at Ohio State University is focused on the use of immunological techniques for rapid diagnosis of these two diseases.

Antibody-producing clones were developed for the causal agent of necrotic ring spot (Leptosphaeria korrae (LK)). Sixteen original clones were subcloned, purified, and screened against LK and non-LK fungi, soil, plant tissue. A single clone with the desired selectivity was increased for bulk antibody production. The final antibody reacted strongly against all verified strains of LK tested (including three from bermuda grass) and did not react significantly with 42 non-LK antigens (including Magnaporthe, Gaummanomyces, plant tissue, soil, and common plant-inhabiting fungi). Final refinements underway are: testing of LK antibodies using field-infected plant material and optimizing assay procedures for routine clinic use.

Similarly, monoclonal antibodies for summer patch (Magnaporthe poae) are under development. The first screen has been completed and indicated that the mouse antibodies react well with the original M. poae strain used for immunization. Subcloning, purification, and extensive screening are underway to acquire antibodies of the desired selectivity.

RATIONALE

The diagnosis of summer patch (Magnaporthe poae) and necrotic ring spot (Leptosphaeria korrae) diseases of Kentucky bluegrass, annual bluegrass, bermuda grass, and other grasses is very difficult with current techniques. Disease symptoms resemble those for other patch diseases and for some abiotic disorders. Isolation of these fungi from infected plant tissue is difficult as is their identification even when growing in a petri plate. New technologies are available that allow the rapid diagnosis of fungal diseases through the use of polyclonal or monoclonal antibodies. The impact of this new approach is already being felt in the area of turf grass management. Antibody kits are in use for detection of Pythium blight, brown patch, and dollar spot.

It is not expected that private companies will produce antibody-based detection kits for necrotic ring spot and summer patch in the near future because 1) these disease complexes are not well understood, 2) the pathogens attack crowns and roots, making assaying more difficult, and 3) the diseases are not as prevalent as dollar spot, brown patch, and Pythium blight.

The primary goal is to provide a clinic tool for diagnosing necrotic ring spot and summer patch diseases. As a research tool the antibodies will allow us to survey the abundance of the pathogens, examine the diversity within the two pathogen populations and to study population dynamics over time in turf grass stands. A future possibility is the development of a rapid assay suitable for use in the field by turf grass managers.

PROGRESS REPORT

SUMMARY OF 1987 ACTIVITIES (condensed from 1987 report)

Leptosphaeria korrae (LK) strain ATCC 56289, and Magnaporthe poae (MP) strain ATCC 60239 were grown in shake cultures of potato-dextrose broth for 2-3 weeks. The resultant fungal mycelia were washed with distilled water, lyophilized, ground to a fine powder in liquid nitrogen, and stored at 4 C until needed for antibody production.

Polyclonal antibodies were developed in a female New Zealand white rabbit against LK. The resultant antisera was highly reactive with LK.

Monoclonal antibodies against LK were produced with the technical assistance of a monoclonal facility in the Department

of Microbiology, Ohio State University (Fig. 1). Eighteen original clones were subcloned, purified, and screened against LK and non-LK fungi, soil, plant tissue, and other common components. The result was 10 clones with good selectivity.

SUMMARY OF 1988 ACTIVITIES

NECROTIC RING SPOT WORK

Selection of Best Clone Against LK: Putative LK strains from 9 states (Table 1) were gathered and grown in shake culture to produce hyphae for antibody clone evaluation. All 58 strains were grown on sterile hard fescue 'Scaldis' seed at 18 C in order to induce the sexual stage. The sexual stage (ascospores) must be seen to verify the identity of putative LK strains. So far, 24 out of 58 strains have produced ascospores characteristic of LK--many of the remaining strains do resemble LK.

The 10 clones were screened and a single clone, 412E4IA9G5 (LK clone), showing the best selectivity was increased for bulk antibody production.

Detailed Screen of LK Clone Against LK: Antibodies from clone 412E4IA9G5 (LKc5) reacted strongly against lyophilized mycelia of all 24 verified strains of LK (including three from bermuda grass) and against all but one of the 34 putative LK strains (Table 1, Fig. 2).

Detailed Screen of LKc5 Against Non-LK Antigens: LKc5 antibodies were tested against a wide array of antigens including Magnaporthe strains (Table 2) and a wide array of organisms commonly associated with turf grass (Table 3). The LKc5 antibodies did not react with any of the Magnaporthe spp. strains and did not react with 42 of 44 antigens (Fig. 3). A weak reaction was noted with strain PV222 (Pythium vanterpooli) and 3375 (Ascochyta sp.).

Lkc5 was determined to be an IgM immunoglobulin, a common type of antibody produced by mammals against fungi. Purification procedures specific for IgM were used to remove non-immunoglobulin proteins (Table 4). The current 3 step assay method requires 24 hr (Table 5).

A paper on this necrotic ring spot work is to be presented at the national American Phytopathological Society Annual meeting in San Diego in November, 1988. A copy of the abstract has been added as an appendix to this annual report.

SUMMER PATCH WORK

Monoclonal antibodies for summer patch (Magnaporthe poae) are under development. Antisera from the six immunized mice has been tested--antibodies from several mice reacted well with the original M. poae strain used for immunization. Spleen cells (antibody-producing cells) from the positive-reacting mice have been fused with myeloma cells (immortal cell line). Cells from the fusions are now being subcloned and tested for antibody production and immortal growth characteristics.

Nine putative strains of Magnaporthe have been collected from 4 states and grown in shake culture for mycelia production. The identities of the cultures are being verified by induction of the perfect stage. The perfect stage is produced by pairing M. poae strains of the opposite mating types 'A' and 'a'. Additional M. poae strains are being collected for a more complete screening procedure.

FUTURE STEPS

NECROTIC RING SPOT WORK

The LKc5 antibodies will be tested for the ability to detect Leptosphaeria korrae in field samples from a variety of locations in Ohio and other states. The clone appears to react well with all verified strains of LK tested to date, including strains from Bermuda grass. Positive reactions occurred with tests of strains of LK growing on agar plates, inoculated plants from the greenhouse, and naturally-infected plants from research plots. Of some concern is the weak but appreciable reaction of the LKc5 antibodies to the non-LK fungal strains PV 211 (Pythium vanterpooli) and 3375 (Ascochyta sp.). Other strains from these fungal groups will be tested for assessment of this situation.

Most of our testing to date has been with culture-grown mycelia and greenhouse inoculated plants. Future work will concentrate on field sampling combined with traditional isolation procedures.

Our experience with use of antibodies (Agri-Diagnostics, Inc.) to monitor Pythium blight epidemics indicates that Pythium populations in plant tissue drops down to non-detectable levels when conditions are unfavorable for pathogen growth. Little is known about the population dynamics of the necrotic ring spot pathogen throughout the year although miscellaneous observations suggest that greatest populations will be detected in the spring and fall. Assay procedures need to be determined through detailed sampling of various plant parts over time.

We will improve the assay procedure to so that results can be

obtained in 3 hrs instead of 24 (Table 5). Purified LKc5 antibody will be conjugated (bound) to an indicator enzyme so that the assay will change from a three to a two step procedure.

SUMMER PATCH WORK

Subculturing, purification, and screening of the summer patch clones will be continued to obtain a monoclonal antibody with good selectivity.

Additional cultures of M. poae will be collected, grown in culture, characterized, for screening and study of the variations present in the pathogen population. Screening is the most time-consuming and critical phase in the development of monoclonal antibodies.

As with the LK system, the next step will be to test the summer patch antibody with growth chamber and naturally-infected grass samples. Several test sites have been identified for field sampling.

NEED FOR ADDITIONAL SUPPORT

A proposal for additional support from the USGA is pending. Support is needed largely because this work is very labor-intensive. The majority of the technical work was done by Ms. V. Wills, a technician under the direction of Drs. Nameth and Shane. Funds from the initial grant have essentially been exhausted. Technician or graduate student help is the greatest need of this project.

In addition, a bottleneck in the research effort has been the bottom-of-the-line ELISA plate reader we have been using. The reader is showing signs of ageing and introduces systematic errors in our assays due to the time needed to read a 96 well plate. The acquisition of a faster plate reader will greatly aid development of the summer patch clones and field evaluation of both summer patch and necrotic ring spot antibodies.

The proposed budgets (I & II) as originally submitted in November 1987 still correctly address the current needs for continuation of the project. We thank you for the support we have received thus far, and for your careful consideration of the new proposal.

CREDITS

Thanks go to Vicki Wills, for her technical assistance. With both the necrotic ring spot and summer patch work the following people provided cultures, comments and/or suggestions:

Dr. Gary Chastagner, Dr. Joe Vargas, Dr. Gail Worf, Dr. Peter Landschoot, Dr. Noel Jackson, Dr. David Grothaus, Dr. Peter Dernoeden, and Dr. Richard Smiley.

Table 1. Suspected and Verified Strains of Leptosphaeria korrae used in tests.

Wisconsin
13-1A = LK22
13-1G = LK26

Minnesota
13-1C = LK23
13-1D = LK24
13-1F = LK25

Patersen, WA
13W-1
13W-3
13W-6
13W-7
13W-9

Kennewick, WA
13W-19
13W-28
13W-32
13W-34
13W-35

Renton, WA
13W-42
13W-43
13W-44
13W-49

Mill Creek, WA
13W-55
13W-62
13W-64
13W-65
13W-66

Spokane, WA
13W-78
13W-80
13W-81
13W-86

Coulee Dam, WA
13W-87
13W-96
13W-99
13W-102
13W-103

Michigan
13-1J = LK28
13W-m1
13W-m5

Pullman, WA
13W-107
13W-109
13W-110

Spokane, WA, Group 1
13W-111
13W-113
13W-114
13W-115
13W-116
13W-126
13W-127
13W-129
13W-140
13W-141

Puyallup, WA
13W-142
13W-144

California
13W-ca1 (Bermuda grass)
13W-ca2 (Bermuda grass)
13W-ca3 (Bermuda grass)

Utah
13W-u84

New York
13W-N114
56289 = N21 = 13-1H = LK27

Colorado Springs, CO
13W-co236
13W-co700

Table 2. Suspected and Verified Strains of Magnaporthe sp. used in tests.

Rhode Island		
12W-ri1 = RI1	Magnaporthe sp.	Turf grass
12W-ri2 = RI2	Magnaporthe sp.	Turf grass
12RI-WR-1-85 = WR-1-85 = ATCC64131	Magnaporthe poae type 'A'	Poa annua L.
12RI-73-1 = ATCC64412	Magnaporthe sp. type 'a'	Poa sp.
New York		
ATCC60239 = 12w-PG57 = 57-84 = Pg57	Magnaporthe sp.	Kentucky bluegrass
ATCC56773 = 60	Magnaporthe sp.	Kentucky bluegrass
12W-pg197	Magnaporthe sp.	
Virginia, Arlington		
12RI-ARL-1	Magnaporthe sp.	Kentucky bluegrass
Columbus, OH		
MP1		Poa annua

Table 3. Non-Leptosphaeria and Non-Magnaporthe Antigens Used in Screen of LK and MP Clones.

Id Code	Identity	Source
ALT1	Alternaria spp.	Turf grass
ALT2	Alternaria spp.	Turf grass
3375	Ascochyta	Kentucky bluegrass
CURV1	Curvularia spp.	Kentucky bluegrass
14-1A	Diaporthe phaseolorum	Soybean
25-1A	Drechslera erythrospila	Turf grass
30-1A	Drechslera poae	Kentucky bluegrass
30-2A	Drechslera catenaria	Turf grass
40-1A	Exserohilum holmii	Turf grass
FUS1	Fusarium sp.	Turf grass
FUS2	Fusarium sp.	Turf grass
1-3A	Fusarium sp.	Turf grass
1-4A	Fusarium tricinctum	Turf roots
1-11E	Fusarium roseum	Kentucky bluegrass
1-13A	Fusarium sp.	Kentucky bluegrass
ZOY-Ggg	Gaeumannomyces graminis var. graminis	Zoysia ssp.
DAR 59042	Gaeumannomyces sp. type 'A'	Kentucky bluegrass
Gg-3410	Gaeumanomyces graminis	Bermuda grass
Gg6186	Gaeumanomyces graminis	Wheat
Gg6286	Gaeumanomyces graminis	Wheat
LEPTO-5	Leptosphaerulina	Alfalfa
GC-2	Gaeumannomyces cylindrosporus	Kentucky bluegrass
Prior	Phialophora graminicola	Kentucky bluegrass
NIGR	Nigrospora	Turf grass
PA1	Pythium aphanidermatum	Unknown
PA243	Pythium aphanidermatum	Turf grass
PG223	Pythium graminicola	Kentucky bluegrass
PG365	Pythium graminicola	Unknown
PT68	Pythium torulosum	Kentucky bluegrass
PT244	Pythium torulosum	Turf grass
PU211	Pythium ultimum	Poinsetta
PV222	Pythium vanterpooli	Bluegrass
6-1C	Rhizoctonia cerealis	Unknown
6-1X	Rhizoctonia cerealis	Turf grass
6-3A	Rhizoctonia solani	Turf grass
6-3B	Rhizoctonia solani	Turf grass
6-3D	Rhizoctonia solani	Turf grass
7-1A	Sclerotinia homeocarpa	Turf grass
7-1F	Sclerotinia homeocarpa	Turf grass
7-1G	Sclerotinia homeocarpa	Turf grass
7-1X	Sclerotinia homeocarpa	Turf grass
TRIC	Trichoderma	Turf grass
G-GH	Kentucky bluegrass leaf tissue	Greenhouse
G-LA	Kentucky bluegrass leaf tissue	Healthy lawn

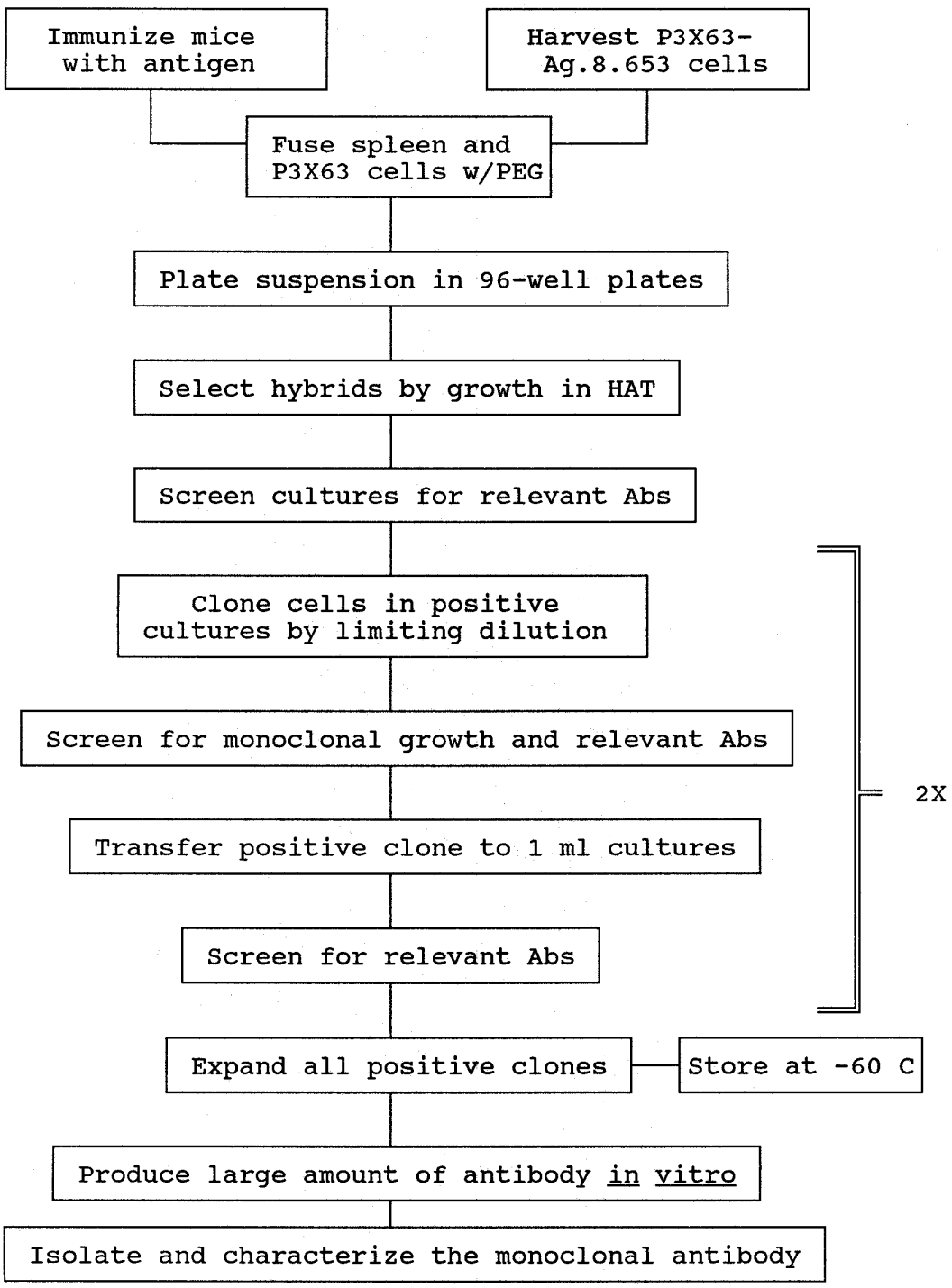
Table 4. IgM Purification Scheme (Jordan 1988).

1. Add sample to 25% NH_4SO_4
2. Centrifuge at 10 K for 10 min. Discard pellet.
3. Bring NH_4SO_4 concentration to 50%
3. Centrifuge at 10 K for 10 min. Save pellet.
4. Resuspend in TBS
5. Dialyse against TBS to remove NH_4SO_4
6. Dialyse against 10 mM Tris no salt pH 7.5
7. Centrifuge at 10 K for 10 min. Save pellet.
8. Resuspend pellet in PBS

Table 5. Two Assay Procedures for Enzyme Linked Immunosorbent Assay (ELISA).

A. Three step procedure
1. Bind antigen (infected plant material) and block assay plate, dry overnight in oven
2. Add LKc50 antibody, followed by wash
3. Add commercial anti-mouse antibody (conjugated with enzyme), wash, and assay for retention of anti-mouse antibody
total time = 24 hrs
B. Two step procedure
The LKc50 antibody has already been bound to plate, blocked, and dried overnight (store in refrigerator until needed)
1. Add antigen (infected plant material), followed by wash
2. Add LKc50 antibody (conjugated with enzyme), wash, and assay for retention of conjugated LKc50 antibody.
total time for steps 1 & 2 = 3 hrs

Figure 1. Procedure for production of monoclonal antibody.



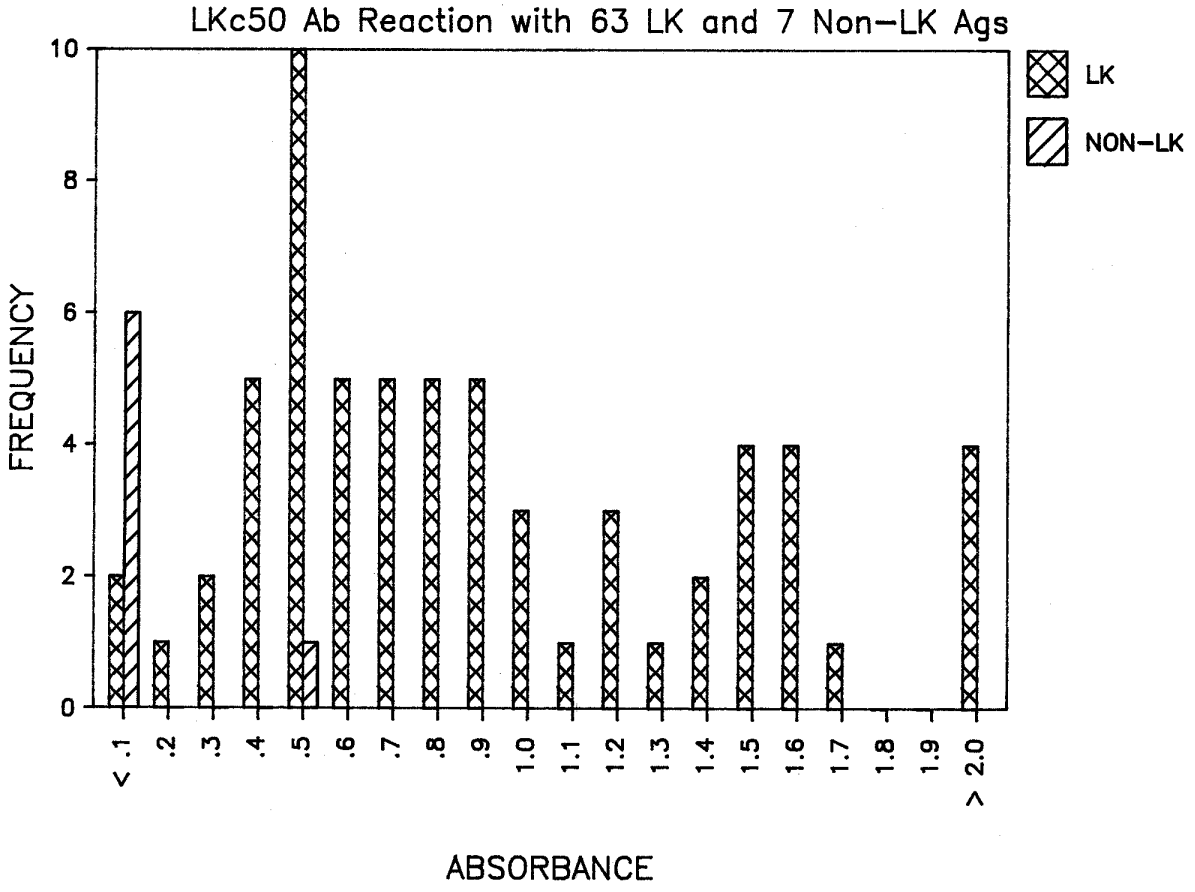


Figure 2. Reaction of clone LKc50 antibodies with 63 Leptosphaeria korrae strains and 7 non-LK fungal isolates. The strengths of the reactions are measured as the absorbance (color intensity) at 405 nm in an ELISA plate test.

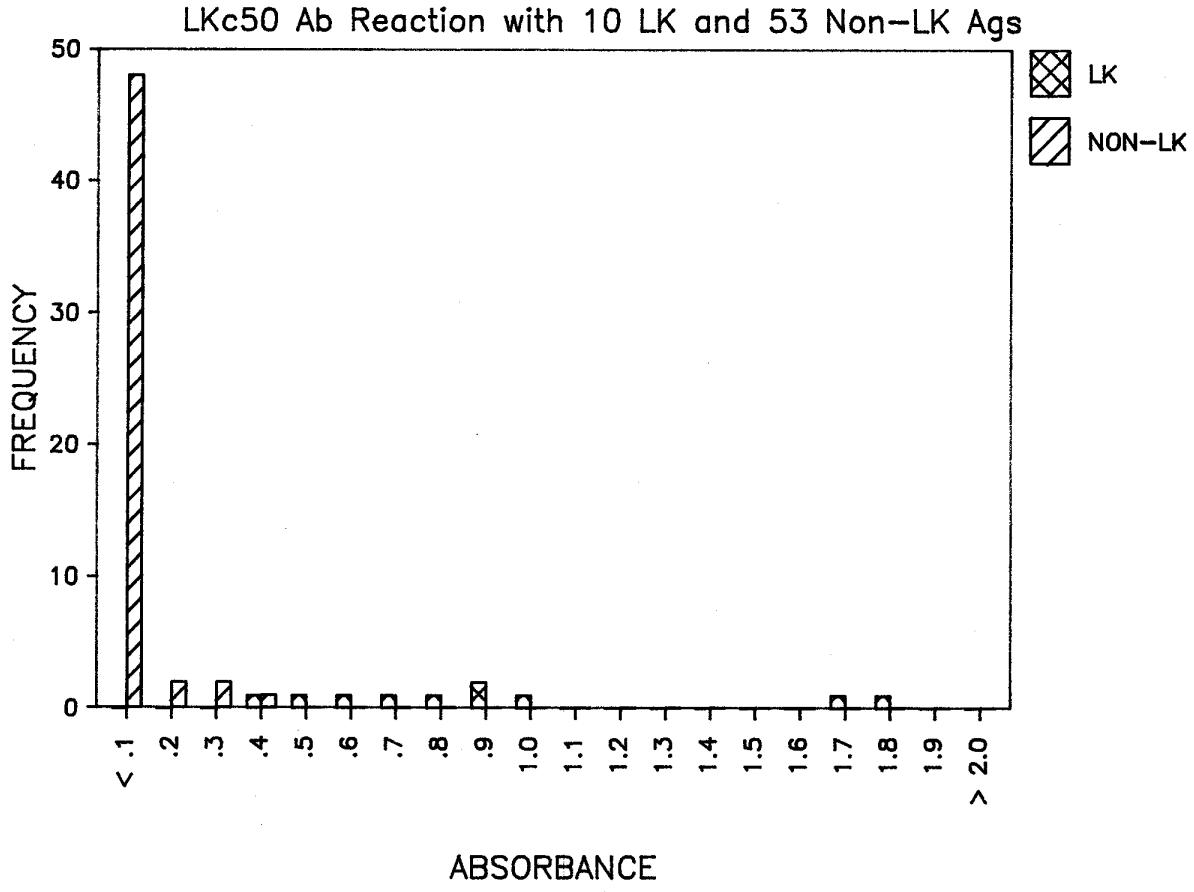


Figure 3. Reaction of clone LKc50 antibodies with 10 Leptosphaeria korrae strains and 53 non-LK fungal isolates. The strengths of the reactions are measured as the absorbance (color intensity) at 405 nm in an ELISA plate test.

APS 1988 Annual Meeting Abstract Form

	Code	Subject
1.	15	Diseases--Ornamentals and turf grass crops
2.	21	Fungal diseases
3.	32	Plant disease detection

W. W. Shane and S. T. Nameth. Monoclonal Antibodies for Diagnosis of Necrotic Ring Spot of Turfgrass. Ohio State University, Columbus

MONOCLONAL ANTIBODIES FOR DIAGNOSIS OF NECROTIC RING SPOT OF TURFGRASS. W. W. Shane and S. T. Nameth, Dept. Plant Pathol., Ohio State University, Columbus, OH 43210

Rapid diagnosis of necrotic ring spot disease of Kentucky bluegrass is hindered by the lack of definitive symptoms and culture characteristics. Verification currently requires the production of the sexual stage--a process that takes > 1 month in culture. Monoclonal antibodies were produced against mycelial homogenates of Leptosphaeria korrae (LK), strain ATCC 56289, for development of a diagnostic test. Spleen cells, from immunized Balb/c mice, were fused with NS-1 myeloma cells and supernatants from resultant hybridoma were screened by indirect ELISA. Eleven lines testing positive against LK56289 were subcloned and tested twice. A subclone was selected exhibiting positive reaction against 11 LK strains representing 5 states and no reaction with various non-LK fungi and non-LK infected plant sap. Non-LK fungi testing negative included among others: Magnaporthe poae, Rhizoctonia spp., Fusarium spp., and Gaeumannomyces spp.

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2 November 1988

Mr. Wiliam H. Bengeyfield
Green Section
United States Golf Association
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Suite 209
Tustin, California 92681
(714) 544-4411

Dear Mr. Bengeyfield:

Enclosed with this letter is the 1988 Annual Research Report for project "Monoclonal Antibodies for Rapid Diagnosis of Summer Patch and Necrotic Ring Spot Diseases of Turfgrass". We have been making very good progress with our work, primarily due to the support received from the USGA.

A paper on the necrotic ring spot work will be presented at the American Phytopathological Society Meeting in November 1988. The support of the USGA will be acknowledged at that time.

Again, we greatly appreciate the support of the USGA.

Sincerely yours:

William W. Shane

William W. Shane
Assistant Professor

cc: Dr. Steve Nameth