REFINEMENT OF THE HOST-PATHOGEN INTERACTION SYSTEM

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The Host-Pathogen Interaction System (HPIS) is an *in vitro* cell selection system developed in conjuction with efforts to obtain creeping bentgrass with resistance to *Rhizoctonia solani*. The HPIS is a unique cell selection technique in that it permits the simultaneous transfer of various substances from a plant pathogenic microorganism to a callus culture during concurrent growth, while avoiding direct physical contact between the organisms. The assembly and application of the HPIS evolved through a series of experiments dating back to 1988. With an understanding of its application for *in vitro* cell selection, we have focused our efforts in 1991 on refining HPIS protocol.

Isolates from the USGA culture collection of *Rhizoctonia* spp., (courtesy of Dr. Phil Colbaugh, Texas A&M University), were co-cultured (concurrently grown) with creeping bentgrass callus in the HPIS. The pathogenic isolates inhibited callus growth and development whereas the non-pathogenic isolates had no effect on callus viability. Studies were conducted to determine effects of various tissue culture media on vigor and pathogenicity of *R. solani*, primarily hormones and energy source concentrations. Various HPIS cultural studies were conducted focusing on length of incubation, duration of concurrent growth-interactions, establishing cultural practices for calli following co-culturing in the HPIS, and examining the persistance of toxicity within the HPIS plates.

Some important questions pertaining to HPIS protocol have been answered by these refinement studies. We know that: 1) pathogenicity at the whole plant level is similarly related to pathogenicity at the cellular level; 2) media components, especially growth hormones and energy sources, play an important role in the pathogenic expression of *R. solani* in the HPIS; 3) we can maximize the use of HPIS plates with successive co-cultures and 4) *R. solani* must incubate at least seven days in the HPIS and there must be a 24 h duration of concurrent growth-interactions between *R. solani* and creeping bentgrass callus before resistant callus can be recovered.

We are rapidly achieving an understanding of how to optimize the HPIS toward our overall and final goal of developing disease resistant variants. Some questions still remain that will be addressed in currently ongoing and future HPIS studies.

ANNUAL PROGRESS REPORT OF RESEARCH

"REFINING THE HOST-PLANT INTERACTION SYSTEM
FOR OBTAINING DISEASE RESISTANT CREEPING BENTGRASS"

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The HPIS 1988-1990:

The initial assembly and application of The Host-Pathogen Interaction System (HPIS) evolved through a series of experiments. We developed the HPIS in conjunction with our efforts to obtain creeping bentgrass callus with resistance to Rhizoctonia solani. Initial experiments concentrated on establishing acceptable cultural practices for each organism. Isolate RVPI of R. solani was grown in the HPIS for 7 or 14 days, at which time callus was plated and co-cultured for an additional 12 days. It was determined that 7 days incubation was sufficient time for RVPI to cover the surface of its growth medium, but 12 days was far too long for the callus to co-culture with RVPI. The callus was unable to produce plantlets when cultured on regeneration medium. Reducing the co-culturing time to three days still resulted in severe callus necrosis. Additional studies focused on a 24, 48, and 72h co-culture. Still no plantlet regeneration occurred, but viable callus appeared to be present. It became evident the HPIS was a potential selection tool for obtaining creeping bentgrass callus tolerant or perhaps resistant to R. solani. The emphasis of research then focused on developing an efficient, reproducible method for analyzing callus viability. A triphenyl tetrazolium chloride (TTC) staining procedure was employed along with a grid sampling system to count viable callus sites within a population of callus that had been subjected to co-culturing with RVPI. This stain is widely utilized to determine viable tissues in damaged seeds and buds. Using TTC stain, callus viability was determined following 8-, 12-, 16-, and 24h of co-culturing with 7 day RVPI cultures. The 24h period of co-culturing was of sufficient duration to yield a desired 25% recovery of viable callus.

Exciting progress on the refinement of HPIS protocol has been accomplished over the past year. The results of each study have taken us one step closer to attaining our final goal, that is the isolation of disease resistant, creeping bentgrass germplasm.

USGA isolate study:

In refining the HPIS, a question arose as to how pathogenicity of R. solani at the whole plant level would compare to that observed in the HPIS at the cellular level? Seven USGA isolates of R. solani and the RVPI isolate of R. solani were used in this evaluation. The HPIS segment of the study consisted of a 24h co-culturing following 7 d of growth by the fungus. That is, R. solani isolates were inoculated and separately grown in the HPIS

for 7 days prior to callus introduction into the HPIS. The callus and isolates interacted (were co-cultured) for 24h at which time callus viability was determined using the TTC stain.
The following table summarizes the pathogenicity of the various isolates of R. solani on Pencross callus and whole plants.

R. solani isolate	Callus Viability[1]	Whole Plant Pathogenicity rating[2]
	- - % - -	
RVPI	23	2.5
R43	86	3.5
R49	81	1.0
R64	78	2.5
R68	100	3.0
R8	95	0.0
R12	99	0.0
R46	100	0.0
CONTROL	100	0.0
LSD (0.05)	9.5	

[1]Callus viability based on TTC stain procedure.
[2]Pathogenicity based on a greenhouse inoculation test scored on a visual scale of 0 to 4; with 0 = nonpathogenic and 4 = highly pathogenic.

The results of this study indicate R. solani pathogenicity at the whole plant level is similarly related to the inhibition of callus viability at the cellular level.

Media studies:

Another aspect of HPIS refinement that needed addressing was the type of media used in the HPIS. A water agar medium is used routinely to culture R. solani, but what influence does the presence of a nutrient-rich tissue culture medium have on R. solani? The following table summarizes effects of tissue culture media on the vigor and pathogenicity of isolate RVPI.

Media Type	Callus Viability[1]
	- - % - -
3MS[2]	34 a[3]
0MS[4]	49 b
water agar	89 c

[1]TTC stain procedure analyzed callus viability following six days of co-culturing in the HPIS.
[2]Tissue culture medium containing 3mg 2,4-D/L.
[3]Letters that differ within columns are significantly different at the P=0.05 level.
[4]Tissue culture medium - no 2,4-D.

These results indicate callus viability was reduced when 2,4-D was present in the tissue culture medium. And while 2,4-D stimulated the vegetative growth of RVPI, the nutrients in the basal medium (OMS) were also stimulatory but to a lesser degree. These findings indicate that as the vegetative growth of RVPI is stimulated, so too is its pathogenicity to the callus.

The next step in media evaluation was to determine the effect of the relative amount of energy source (sucrose content) on RVPI pathogenicity. The optimum level of sucrose in Murashige and Skoog (MS) medium is 30g/L which was considered 100% sucrose in this study. The MS media used in this study contained 3mg 2,4-D/L. The sucrose levels were as followed: 0, 25, 50, 75, and 100%. The study was conducted by co-culturing callus for 24h against 7 d RVPI growth in the HPIS. Callus viability was determined using the TTC stain. Only 2% viable callus was recovered from the treatments containing sucrose whereas callus co-culturing in the absence of sucrose lost only 26% of its viable population. It appears when sucrose and 2,4-D are present in the tissue culture medium, vegetative growth of RVPI is accelerated. When RVPI is stimulated to produce copious amounts of vegetative material, the level of pathogenicity is very high. Knowing this, we can manipulate the level or degree of pathogenicity of R. solani somewhat by the hormone and strength of the energy source in the HPIS media. This principal must be considered as we continue to refine the HPIS.

Leaf blade study:

Penncross leaf blades are employed in the HPIS as a natural substrate in conjunction with water agar to support the growth of R. solani. Initially, the leaf blades were autoclaved prior to use in the HPIS however, we wanted to determine whether live (non-autoclaved) sterile leaf blades would be more or less beneficial in promoting growth and pathogenicity of R. solani. Our findings indicate no significant differences between autoclaved and non-autoclaved leaf material. The RVPI isolate reduced callus viability by 80% in both treatments.

Callus rinse study:

The objective of this study was to determine whether rinsing the co-cultured callus could wash off toxic substances, thus increasing the survival rate of the callus population. Using the standard 7 d RVPI growth and 24 h co-culturing of creeping bentgrass callus, callus from randomly selected plates was thoroughly rinsed with liquid MS medium and then plated onto regeneration medium. Other callus was transferred directly to regeneration medium without washing. The number of regenerated plantlets was determined for each treatment. The rinsed callus gave rise to one plantlet per culture as did the non-rinsed callus. The control treatments (lacking RVPI) averaged 28 plantlets per culture, regardless of washing with MS medium. This indicates there is no benefit in rinsing the calli following co-cultures with RVPI. The toxic substances are being absorbed by the calli and the susceptible calli are being killed. The surviving or resistant

calli are somehow adapting themselves to the antagonistic effects of these substances and remaining viable.

Repeated use of the HPIS plates:

Once RVPI is established in the HPIS, we wanted to determine how long RVPI can maintain its pathogenicity following successive co-cultures with creeping bentgrass callus. The study involved two types of tissue culture media, 3MS and water agar. Three successive 24h co-cultures were conducted using the same HPIS plates. Again callus viability was dependant on the type of tissue culture medium. Callus cultured on 3MS had 26% viability, whereas callus viability was 68% when cultured on water agar. Our main objective, however, was to examine the persistence of toxicity within the HPIS plates. No differences in callus viability were noted within the 3MS or water agar containing HPIS plates when they were reused for a successive number of co-cultures. These results are important because recycling the HPIS plates for at least two additional co-culturings with callus (before the RVPI and media become exhausted) will assist us in our efforts to limit the expense and labor involved in these studies. It will also enable us to screen large populations of callus for disease resistance in shorter period of time.

Immediate co-culture study:

This study was conducted to determine at what point in time the callus population is reduced to 25% viability when RVPI and callus are introduced simultaneously into the HPIS. Callus viability was determined every 24h over a period of 2 to 9 days following co-culture initiation. The tissue culture medium employed was 3MS. After 2d of co-culturing, about 85% of the callus remained viable. The RVPI had not vegetatively covered the surface of its water agar growth medium. On the seventh day of co-culture the viable callus population had declined to 28%. On the ninth and final co-culture day, only 8% viable callus remained. There is a clear indication from this study that to a certain extent, the more vegetatively mature the RVPI is, the more pathogenic it becomes to the callus. This same trend was observed in the media studies. However, it has been suggested that when exposing callus to a selection agent, a gradual exposure may prove beneficial by promoting a higher frequency of cell mutations. These mutations occur in response to there environment and because of somal clonal variation. When creeping bentgrass callus undergoes co-culturing concurrently with the growth and development of the fungus, there may be a greater frequency of adaptation by the callus to the pathogenic effect of RVPI. This would be in contrast to the delayed co-culture where the callus is exposed to a high level of toxic substances for a short period of time after fungal growth and development has already occurred. In this later system, toxic levels appear to accumulate sufficiently to destroy 75% of a callus population during 24 h of co-culturing exposure.

This sudden and massive exposure may be too severe. Future studies are planned to compare callus quality following immediate and delayed co-cultures.

RVPI incubation study:

RVPI growth and development times were varied from 24 h to 10d in order to determine the exact incubation time at which a 75% reduction of viable callus would be observed. At 24h intervals callus was plated and co-cultured with RVPI for 24h. Callus viability was determined immediately afterwards using the TTC stain. Following three days of incubation, RVPI inhibited callus viability by 50%. Following seven days of incubation callus viability was reduced 74%. And after RVPI growth for 10 d, callus viability was reduced by 80%. The information gathered from this study follows the same trend as that observed in other co-culture studies that have been discussed previously. After approximately seven days of RVPI growth in the HPIS, creeping bentgrass callus mortality approaching 75% can be obtained in a 24 h co-culturing. We now have a much better understanding of the development of RVPI in the HPIS, and at what growth stage we can optimize its selective, lethal interaction with creeping bentgrass callus toward isolation of resistant callus.

Conclusions:

Some very important questions pertaining to HPIS protocol have been answered by these refinement studies. We know that: 1) pathogenicity at the whole plant level is similary related to pathogenicity at the cellular level; 2) media components play an important role in the pathogenic expression of RVPI in the HPIS; 3) we can maximize the use of HPIS plates with successive co-cultures and 4) RVPI must grow for at least seven days in the HPIS before a 24 h co-culturing exposure will kill about 75% of the callus and render a 25% recovery of resistant callus material. Another way to interpret this is RVPI must be at a certain stage of morphological development before an adequate concentration of toxic substances are present to inhibit 75% of the callus population.

We are rapidly achieving an understanding of how to optimize the HPIS toward our overall and final goal of developing disease resistant variants. However, some questions still remain that will be addressed in currently ongoing and future HPIS studies.