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## *RESEARCH PAPERS*

# Interaction between *Pyricularia oryzae*, four *Helminthosporium* species and *Curvularia lunata* in rice leaves

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**Summary.** The interaction between six fungal parasites of rice: *Pyricularia oryzae*, *Helminthosporium oryzae*, *H. sativum*, *H. spiciferum*, *H. australiensis* and *Curvularia lunata* was studied quantitatively by a modified plant ecology technique known as the de Wit replacement series. Each fungus was inoculated alone or in combination with one of the other five fungi in various proportions into rice plants under experimental conditions. Leaves developing lesions were harvested and incubated in a moist chamber. The yield of each fungus was its conidial production on the rice leaves. The artificial inoculations indicated that interactions between the pathogens in the mixture could be beneficial, antagonistic, or null. Interspecific interaction (i.e. antagonism) occurred in the majority of paired combinations (*H. oryzae* + *P. oryzae*; *H. sativum* + *H. spiciferum*, *H. australiensis*, *C. lunata* or *P. oryzae*; *H. australiensis* + *H. spiciferum*, *C. lunata* or *P. oryzae*; and *P. oryzae* + *C. lunata*). The relative yield total (RYT) lines were significantly lower than the expected value, which is 1. The RYT lines were concave upward, revealing a beneficial effect of one or both pathogens on the other, when *H. oryzae* was in mixture with *H. sativum* or *H. spiciferum*. A null effect between fungi occurred in four combinations (*H. oryzae* + *H. australiensis* or *C. lunata*; *H. spiciferum* + *C. lunata*; and *P. oryzae* + *H. spiciferum*) showing that with these combinations inter- and intraspecific competitions were equal in intensity. Thus, the de Wit replacement series technique indicated that it was possible to quantify the interaction between all the pathogenic fungi tested.

**Key words:** *Oryza sativa*, de Wit, replacement series, pathogenic fungi, antagonism, leaf spot.

## Introduction

In a natural rice-growing environment, pathogenic micro-organisms rarely grow in isolation from each other, and the success of one organism may be dependent upon the metabolic activity of another. For example, it has been shown that plants attacked by one pathogen thereafter become predisposed to attack by a second, unrelated pathogen (Yarwood, 1959). *Pyricularia oryzae* Cavara, *Helminthosporium oryzae* Breda de Haan, *H. spiciferum* (Bainier) Nicot, *H. australiensis* Bugnicourt, *H. sativum* Pammel, King & Bakke and *Curvularia lunata* (Wakk.) Boedijn all cause leaf spots on rice, and more than one can be isolated from foliar lesions on the same rice plant (Benkirane *et al.*, 1995). All these pathogens are found in Morocco (El Oirdi *et al.*, 1995; Bouslim *et al.,* 1997; Hassikou *et al.,* 1997; Ennaffah *et al.,* 1999; Ouazzani

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Touhami *et al.*, 2000). In this situation, an assessment of direct losses from a given pathogen on the basis of symptoms in the field is difficult, since interaction with other pathogens may affect disease intensity (Ouazzani Touhami, 2001).

Competitive interactions in a broad sense are fundamental to our knowledge of the ecology of plant pathogenic fungi (Rayner and Webber, 1984). Interactions between organisms have been studied using a number of techniques, including inhibition tests *in vitro* (Deacon, 1976; Skidmore and Dickinson, 1976), microscopic examination of hyphal interactions (Ikediugwu and Webster, 1970; Dennis and Webster, 1971; Deacon, 1976; Skidmore and Dickinson, 1976; Willingale and Mantle, 1987), and germ tube inhibition (da Luz and Bergstrom, 1987; Mercier and Reeleder, 1987). Competition can also be assessed by measuring the biomass of the interacting fungi. However, methods for hyphal biomass measurement have proved unreliable and in most cases not specific for the fungus under examination (Jones and Mollison, 1948; Frankland, 1975).

In the present study, the replacement series of de Wit was used to determine whether interactions occurred between any of six pathogenic fungi (*P. oryzae*, four *Helminthosporium* species and *C. lunata*) on rice leaves under controlled experimental conditions.

The de Wit replacement series (de Wit, 1960) is one of the most widely used designs for the study of competitive interactions (de Wit *et al.,* 1966; Harper, 1977; Silvertown, 1982). In replacement series studies, the proportions of two microbial strains in a mixture are varied, while the total population density of the mixture is maintained constant. The relative yields (RYs) of two strains as well as their relative yield total (RYT) are analysed as functions of the varying proportions of the strains. RY is the reproductive output of a strain in the mixture relative to what its output would be if it were alone at the same total population density, and RYT is the sum of the RYs of the two strains.

In the traditional interpretation of the de Wit curves, the null hypothesis states that inter- and intra-strain competitive abilities are equal, so that the expected RY of each strain in a mixture is equal to that strain's proportion in the mixture. In the null hypothesis the expected relationship between strain frequency and RY in the mixture is linear, and the expected RYT is 1. If the curve for the observed RY of a strain plotted against its frequency in a mixture lies above the null straight line, the traditional interpretation is that the yield per individual of this strain is more strongly inhibited by other individuals of the same strain than by individuals of the other strain. Conversely, a RY curve that falls below the null straight line indicates that this strain is more strongly inhibited by the other strain than it is by itself.

## Materials and methods

### Plant material

Rice seeds from the Arco variety were disinfected by soaking in 5% sodium hypochlorite for 10 min and washed 3 times with sterile distilled water. Treated seeds were dried with filter paper, placed in Petri dishes on water-soaked cotton and incubated at 28°C in the dark. After 72 h, seedlings were planted in plastic pots containing black soil obtained from Maamora forest (3 plants per pot), transferred to a greenhouse and watered with tap water at regular intervals until the 4- to 5-leaf stage. Thereafter, the plants were transported to the laboratory for inoculation according to the different treatments.

### Fungal inoculum and inoculation

Strains of *H. oryzae* and *H. spiciferum* were obtained from foliar lesions of the rice variety Triomphe. *H. sativum*, *H. australiensis* and *C. lunata* strains were collected from lesions of the varieties Bahja, Samar and Kenz respectively. *P. oryzae* was obtained from seeds of the Arco variety.

*Pyricularia oryzae* was cultivated on a prune-agar medium (prune, 40 g; agar-agar, 20 g; distilled water, 1000 ml); *H. oryzae* and *H. sativum* on a rice flour medium (rice flour, 14 g; yeast extract, 4 g; agar-agar, 15 g; distilled water, 1000 ml); and *C. lunata*, *H. spiciferum* and *H. australiensis* on a PDA medium (potato dextrose, 40 g; agar-agar, 15 g; distilled water, 1000 ml).

Dishes inoculated with *P. oryzae* were incubated at 28°C for 7 days in the dark, and for the further 3 days at the same temperature under continuous fluorescent lighting. Cultures of *C. lunata*, *H. spiciferum* and *H. australiensis* were incubated at 28°C in the dark, while *H. oryzae* and *H. sativum* cultures were maintained at 28°C under continuous fluorescent lighting.

After cultures of *H. oryzae* were incubated for 15 days and the other 5 fungal species for 10 days, conidia were collected in sterile water and agitated in a vortex for 30–60 s. Conidia suspensions were filtered through muslin to eliminate mycelial fragments. Conidia concentrations were quantified microscopically, and adjusted to a concentration of $10^5$ conidia $ml^{-1}$ with sterile distilled water containing 0.02% Tween 20 and 0.5% gelatin.

Conidial suspensions were sprayed with an atomiser onto the rice leaves. Each fungus was spread either alone or in combination with one of the other five fungi in various proportions (0 first fungus:100 second fungus; 25:75; 50:50; 75:25; 100:0) and at a final concentration of $10^5$ conidia $ml^{-1}$. After each inoculation, the plants were kept in the laboratory for 48 h under black plastic bags to maintain a high relative humidity before they were returned to the greenhouse.

Three replicates (3 pots) were used for each treatment. Each treatment represented an inoculation with one fungal proportion of each fungal pair, and there were 5 proportions per fungal pair.

### Fungal yield

Seven days after inoculation, the average number of conidia produced per leaf was determined using the technique of Hill and Nelson (1983).

Thirty leaves per fungal combination (6 per treatment) with lesions were harvested from the inoculated rice plants, cut into 3–4 pieces of about 1 cm long and put into glass Petri dishes (90 mm) on filter paper soaked with sterile water (1 leaf per dish). The leaf pieces were incubated at 28°C under continuous fluorescent lighting.

After 4 days, pieces of a third leaf were placed in a test tube containing 1 ml water and agitated by a vortex mixer for 2 min. The spores of each pathogen were counted using a haemacytometer (10 counting for each sample). The fungal yield values were defined as the conidial production of each fungus in the rice leaves.

The RY of each pathogen (yield in mixture divided by the yield of a single inoculation) was calculated and then plotted against the input ratios (100, 75, 50, 25 and 0). The RY of each pathogen when present alone (100%) was equal to 1, and the other RYs were calculated with reference to this at each input ratio. The sum of the RYs of two fungi gave the relative yield total (RYT) for those fungi.

### Statistical analysis

The data were analysed by the paired-comparison t-test ($P<0.05$), which compares observed RYs with expected RYs (0.75, 0.5 and 0.25) using a null-hypothesis model in which interspecific competition and intraspecific competition are equal. The observed RYTs were compared with the expected RYT (1) using a nonparametric sign test. The shape of the RYT line reflected the interaction between the two pathogens in the mixture.

## Results

The RY and RYT lines for the interactions between the six pathogenic fungi of rice mixed at different proportions are shown in Figures 1, 2 and 3.

The increase in the RYs of *H. oryzae* in mixture with *H. sativum* appeared to be compensated by a lowering of the RYs of *H. sativum*. The RYs of *H. oryzae* did not differ significantly from the expected RYs, but in the *H. oryzae* + *H. spiciferum* mixture the RY of *H. spiciferum* increased (Table 1). The observed RYTs in these two mixtures were significantly greater than 1, the expected RYT value (Table 2). The concave upward RYT lines in both cases (Fig. 1A and B) were indicative of a beneficial effect of one or both pathogens on the other.

The paired-comparison t-tests (Table 1) revealed the absence of a difference between the observed RYs and the expected RYs in the following combinations: *H. oryzae* + *H. australiensis* (Fig. 1C), *H. oryzae* + *C. lunata* (Fig. 1D) and *H. spiciferum* + *C. lunata* (Fig. 3A). The observed RYs of *P. oryzae* decreased slightly whereas that of *H. spiciferum* increased in the *P. oryzae* + *H. spiciferum* combination (Fig. 3B). In the case of *H. oryzae* + *H. australiensis*, 5 of the observed RYTs were smaller and 4 were greater than the expected RYT of 1; the observed RYTs of the *H. oryzae* + *C. lunata* combination were consistently lower than the expected RYT; 4 RYTs were above 1 and 5 below when *H. spiciferum* was combined with *C. lunata* or *P. oryzae* (Table 2). In these four combinations, no significant deviation of any RYT lines was observed; in the traditional interpretation of the de Wit curves this means that the interspecific and intraspecific competition abilities were equal.

In the combination *H. oryzae* + *P. oryzae* (Fig. 1E) and in the combinations of *H. sativum* + *H. spiciferum* (Fig. 2A), *H. australiensis* (Fig. 2B), *C. lunata* (Fig. 2C) and *P. oryzae* (Fig. 2D) the RYs for

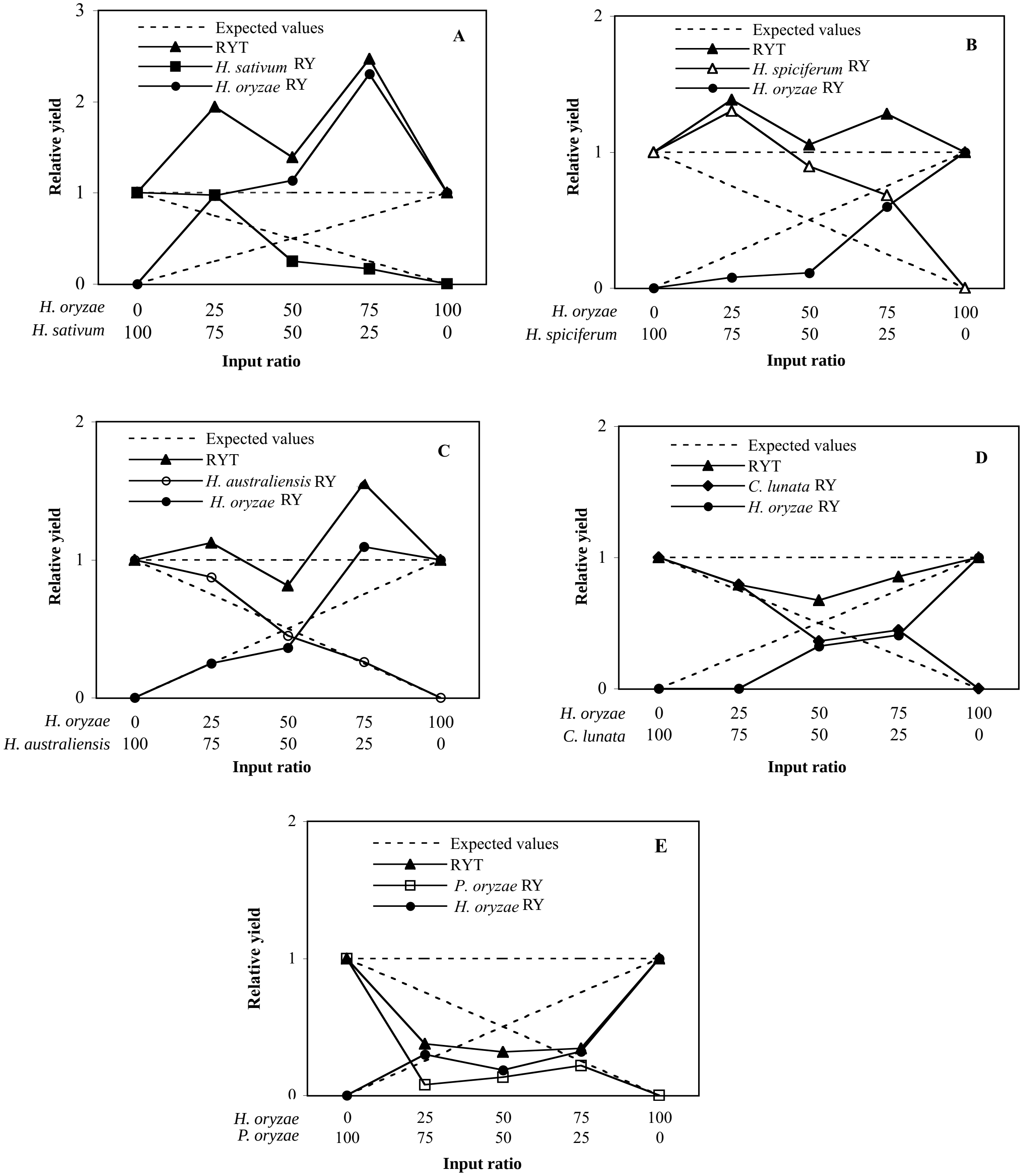

Fig. 1. Relative conidia production on rice leaves inoculated with *H. oryzae, H. sativum, H. spiciferum, H. australiensis, C. lunata, P. oryzae,* alone and in combination, resulting from varied input ratio. RYT, relative yield total; RY, relative yield of each species; Expected values (dotted lines) are reference values indicating equal inter- and intraspecific competition.

Table 1. Results of artificial inoculation on rice leaves of each fungus combined with one of the other five fungi in various proportions (25:75; 50:50; 75:25) comparing observed relative yield (RY) values of each species with expected values.

| Fungal combination | T-test [a] | | |
|---|---|---|---|
| | Below[b] | NS[b] | Above[b] |
| *Helminthosporium oryzae* | | 1 | 2 |
| *H. sativum* | 2 | | 1 |
| *H. oryzae* | 1 | 2 | |
| *H. spiciferum* | | 1 | 2 |
| *H. oryzae* | | 3 | |
| *H. australiensis* | | 3 | |
| *H. oryzae* | | 3 | |
| *Curvularia lunata* | | 3 | |
| *H. oryzae* | 2 | 1 | |
| *Pyricularia oryzae* | 3 | | |
| *H. sativum* | 1 | 2 | |
| *H. spiciferum* | 1 | 2 | |
| *H. sativum* | 2 | 1 | |
| *H. australiensis* | 2 | 1 | |
| *H. sativum* | 3 | | |
| *C. lunata* | 2 | 1 | |
| *H. sativum* | 3 | | |
| *P. oryzae* | 2 | 1 | |
| *H. spiciferum* | | 3 | |
| *H. australiensis* | 3 | | |
| *H. spiciferum* | | 3 | |
| *C. lunata* | 1 | 2 | |
| *H. spiciferum* | | 1 | 2 |
| *P. oryzae* | 2 | 1 | |
| *C. lunata* | | 3 | |
| *H. australiensis* | 2 | 1 | |
| *P. oryzae* | 3 | | |
| *H. australiensis* | | 3 | |
| *C. lunata* | | 2 | 1 |
| *P. oryzae* | 3 | | |

[a] The t-test ($P<0.05$) was used to test the hypothesis that the difference between the observed relative yield (RY) and expected value was equal to 0.

[b] Values are the number of points 'above', not significantly different from (NS), or 'below' the expected points.

both fungi were less than expected (Table 1). The RYs of *H. australiensis* underwent a reduction when combined with *H. spiciferum* (Fig. 2E) and with *C. lunata* (Fig. 3C), but the RYs of these last two fungi combined did not differ significantly from those expected for them (Table 1). When *P. oryzae* was combined with *H. australiensis* (Fig. 3D) or with *C. lunata* (Fig 3E), the RYs for *P. oryzae* were lower but those for *H. australiensis* and *C. lunata* did not differ significantly from the expected RYs (Table 1). The observed RYTs were less than 1 in the nine combinations (Table 2). Thus the concave downward RYT lines indicated that interspecific competition was stronger than intraspecific competition.

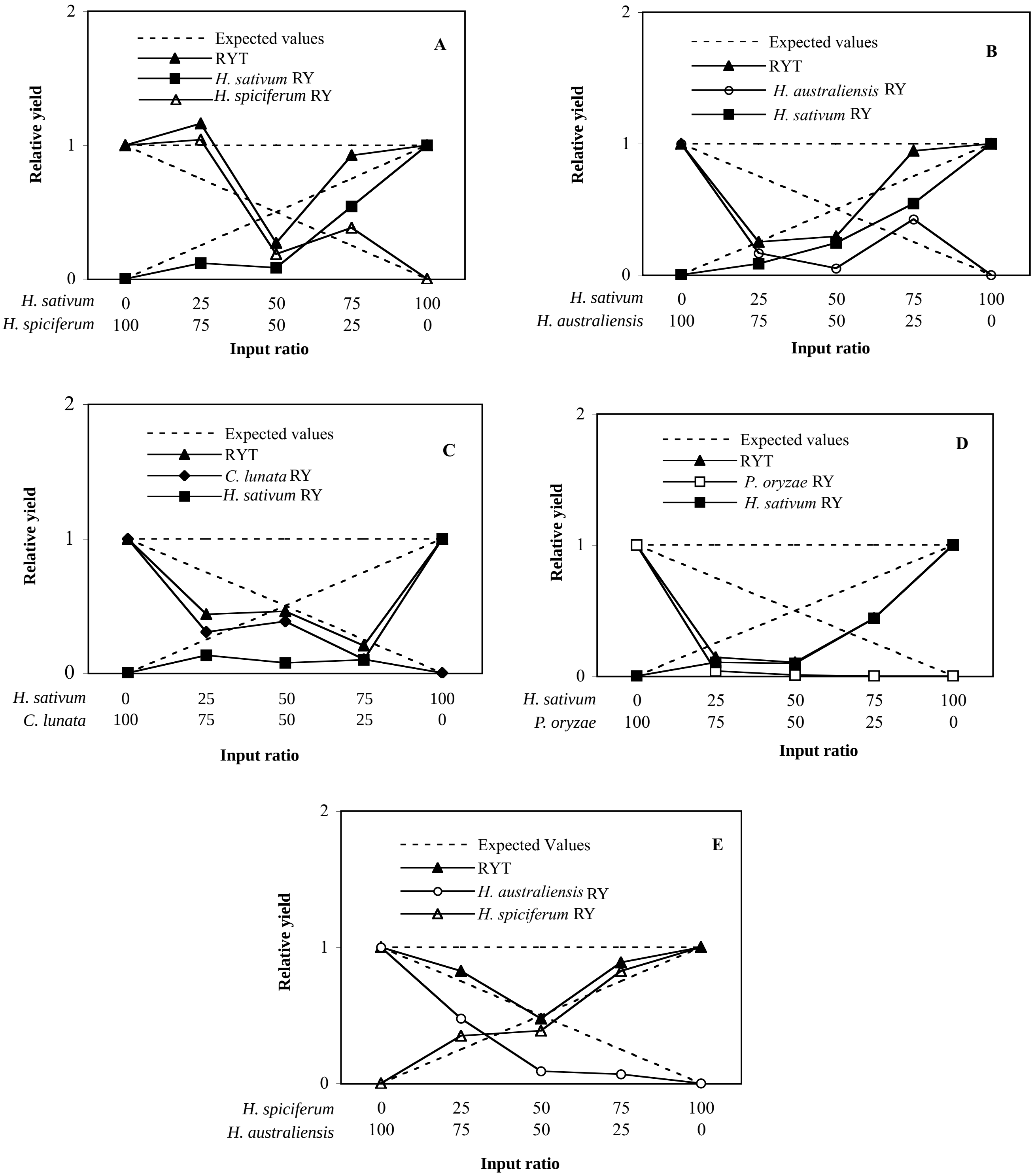

Fig. 2. Relative conidia production on rice leaves inoculated with *H. sativum, H. spiciferum, H. australiensis, C. lunata, P. oryzae*, alone and in combination, resulting from varied input ratio. RYT, relative yield total; RY, relative yield of each species. Expected values (dotted lines) are reference values indicating equal inter- and intraspecific competition.

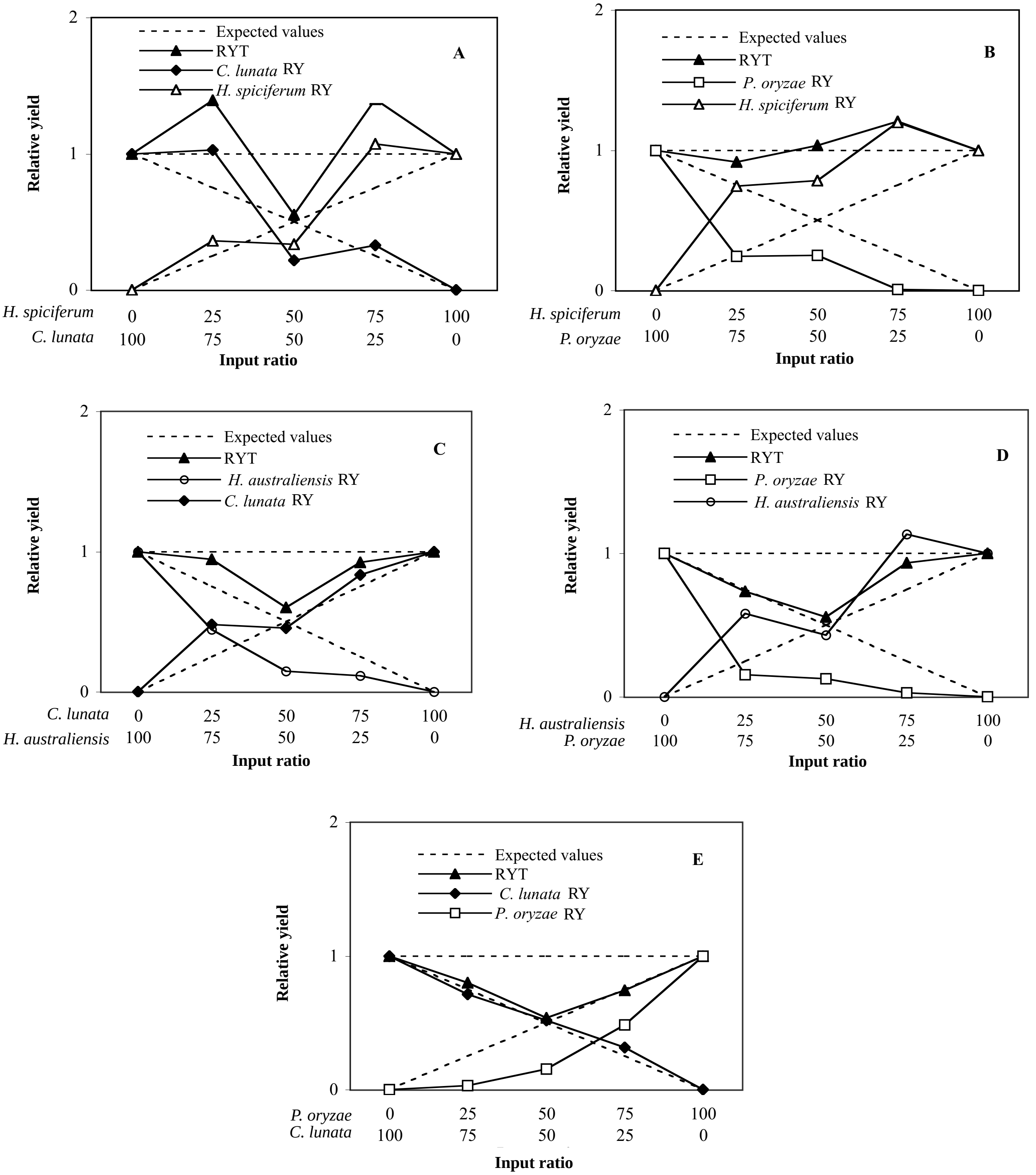

Fig. 3. Relative conidia production on rice leaves inoculated with *H. spiciferum, H. australiensis, C. lunata, P. oryzae*, alone and in combination, resulting from varied input ratio. RYT, relative yield total; RY, relative yield of each species; Expected values (dotted lines) are reference values indicating equal inter- and intraspecific competition.

Table 2. Artificial inoculation with various combinations of pathogenic fungi on rice comparing observed relative yield total (RYT) with expected RYT (i.e. 1).

| Fungal combination | Observed RYT[a] | | |
|---|---|---|---|
| | Less than 1 | Equal to 1 | Greater than 1 |
| *Helminthosporium oryzae* + *H. sativum* | | | 9 |
| *H. oryzae* + *H. spiciferum* | 2 | | 7 |
| *H. oryzae* + *H. australiensis* | 5 | | 4 |
| *H. oryzae* + *Curvularia lunata* | 6 | 1 | 2 |
| *H. oryzae* + *Pyricularia oryzae* | 9 | | |
| *H. sativum* + *H. spiciferum* | 6 | | 3 |
| *H. sativum* + *H. australiensis* | 7 | | 2 |
| *H. sativum* + *C. lunata* | 9 | | |
| *H. sativum* + *P. oryzae* | 9 | | |
| *H. spiciferum* + *H. australiensis* | 7 | | 2 |
| *H. spiciferum* + *C. lunata* | 4 | | 5 |
| *H. spiciferum* + *P. oryzae* | 4 | | 5 |
| *C. lunata* + *H. australiensis* | 6 | | 3 |
| *P. oryzae* + *H. australiensis* | 7 | | 2 |
| *C. lunata* + *P. oryzae* | 9 | | |

[a] The nonparametric sign test, with $P<0.05$, was used to determine the number of RYT observations that were less than, equal to, or greater than the expected value (i.e. 1).

## Discussion

Several recent studies have shown that competitive interactions can be quantified by conidial production of the fungi on the host plants (Adee *et al.*, 1990; Zitko and Timmer, 1994; Klepzig, 1998; Newton *et al.*, 1998; Timmer *et al.*, 1998; Nolan *et al.*, 1999). The de Wit plots describe these interactions. Moreover, spore production represents the total effect that all components of host resistance have on pathogen multiplication, and is therefore the most useful measure of such interactions (Johnson and Taylor, 1976). The de Wit analysis here used spore counts to quantify the competitive interactions between various pathogenic fungi during their colonisation of rice leaves.

With a majority of combinations (*H. oryzae* + *P. oryzae*; *H. sativum* + *H. spiciferum*, *H. australiensis*, *C. lunata* or *P. oryzae; H. australiensis* + *H. spiciferum*, *C. lunata* or *P. oryzae*; and *P. oryzae* + *C. lunata*), RYT lines were significantly lower than expected, i.e. concave downward, indicating a significant interspecific interaction between the combining species. This indicates strong antagonism, such as in allelopathy (Harper, 1977).

By contrast, when *H. oryzae* was combined with *H. sativum* or *H. spiciferum*, the RYT lines were significantly greater than expected (i.e. concave upward). A concave upward RYT line indicates a beneficial effect of one of the pathogens on the other or on itself ,9h]e *et al.*, 1990).

The absence of significant deviation of the RYT lines in four of the combinations (*H. oryzae* + *H. australiensis* or *C. lunata*; *H. spiciferum* + *C. lunata* and *P. oryzae* + *H. spiciferum*) could be an indication that the interspecific and intraspecific competitions were equal in intensity according to the traditional interpretation of the replacemenząseries (de Wit *et al.*, 1966; Harper, 1977; Silvertown, 1982), with a null effect between the fungi.

There have been several criticisms of the de Wit analysis. One is that the typical de Wit replacement series involves only one total density, and thus fails to take into account the expected density-dependence of competition (Firbank and Watkinson, 1985; Snaydon, 1991). It is also reported that effects of changes in the density of one organism (strain) mask the effects of changes in the density of the other, making it impossible to fully describe the reproductive dynamics in combination as a function of the densities of each organism individually (Snaydon, 1991). Recently, Newton (1996) identified another limitation in the de Wit model to quantify competitive interactions. De Wit curves constructed for pathogens, or any organisms whose life cycles have multiple stages during which competitive interactions occur, may not correspond to traditional expectations for particular combinations of intra- and interstrain interactions in a system. Specifically, RY curves may deviate from the null lines because of differences in ecological traits between organisms in mixed populations, other than competitive ability (e.g. carrying capacity, infection efficiency) or they may deviate from the null line in unexpected patterns when competitive differences do exist. Nevertheless, Newton *et al*. (1998) concluded that deviations from expected null RYT straight lines are produced only by differences between intra- and interstrain competition, so that a significant deviation from the null RYT line is good evidence that intra- and interstrain competition differ significantly for at least one of the competing strains. Thus, the significant deviation from the null straight lines observed in this study was a good indicator that there was an interaction between all the pathogenic fungi tested with the exception of *H. oryzae* + *H. australiensis* or *C. lunata*, and *H. spiciferum* + *C. lunata* or *P. oryzae*. However, the de Wit plots do not offer substantial insight into the mechanisms behind the relative reproductive outputs in coexisting pathogens strains (Newton *et al*., 1998).

There are additional factors that may influence the competitive interactions between a plant host and a pathogenic fungus. The co-occurrence of *Pyrenophora tritici-repentis* and *Septoria nodorum* on wheat leaves may induce the secretion of host metabolites toxic to one or both of the fungi (Adee *et al*., 1990). The degree of resistance of the host may also affect infection and/or colonisation processes and, consequently, fungal yield. According to Kuč (1972) a host may become resistant to a pathogen after infection with the conidia from another pathogen. The co-occurrence of other micro-organisms (saprophytic fungi, bacteria, yeasts) on the leaf surface may also influence infection or colonisation of the leaves by a given fungus (Blakeman and Fokkema, 1982). Indeed, competitive interaction may even occur when essential metabolites for micro-organism growth are relatively deficient (Alabouvette *et al*., 1983; Alabouvette and Davet, 1985).

The present experimental study showed the existence of interactions between the majority of six foliar parasites of rice, *P. oryzae, H. oryzae*, *H. sativum*, *H. spiciferum*, *H. australiensis* and *C. lunata*, all of which produce conidia on rice leaf lesions. A significant increase in inoculum from one parasite should have an impact on the disease cycle caused by others. This makes it all the more important to bear in mind that all these pathogenic fungi may interact with each other under natural field conditions.

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