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Field and laboratory screening of anthurium cultivars for resistance to foliar bacterial blight and the induced activities of defence-related enzymes

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ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*) is the most destructive disease of ornamental anthurium. In the present study, foliar resistance of 21 anthurium cultivars were assessed under shaded field and laboratory conditions by injection inoculation of 3×10^8 cfu/ml *Xad*; disease severity was evaluated using a pretransformed rating scale after symptoms survey. Then six selected cultivars with different resistance levels were evaluated for the induced activities of six defence-related enzymes. The obtained results indicated that the same cultivar shared identical resistance under both conditions, but there was a great variation among the cultivars. Anthurium cv. Pink Champion and Manaka showed the highest resistance, and five other cultivars were highly susceptible. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD) and phenylalanine ammonia-lyase (PAL) in the resistant cultivars increased much faster and reached much higher peak levels than those in susceptible cultivars. Further analyses revealed that the relative resistance index (RRI) significantly positively correlated with the activities of SOD, APX, POD and PAL, but not with catalase (CAT) and polyphenol oxidase (PPO), suggesting that early rapid accumulation of SOD, APX, PAL and POD might be an important mechanism of defence against *Xad* and could serve as one of the valuable physiological indices for the prediction of BB resistance in anthurium germplasm. Consequently, the identified resistant cultivars and the induced defence enzymes will facilitate the phytopathological research and enhance blight resistance selection in future breeding.

Keywords: anthurium (*Anthurium andreanum* Lind), antioxidative enzymes, bacterial blight, enzyme activity, *Xanthomonas axonopodis* pv. *dieffenbachiae*

Abbreviations:

APX – ascorbate peroxidase, BB – Bacterial blight, CAT – catalase, CFU – colony forming units, dpi – days post-inoculation, DSI – disease severity index, PAL – phenylalanine ammonia-lyase, POD – peroxidase, ROS – reactive oxygen species, RRI – relative resistance index, SOD – superoxide dismutase, *Xad* – *Xanthomonas axonopodis* pv. *dieffenbachiae*

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INTRODUCTION

In the last three decades, bacterial blight (BB) caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*) has been the most devastating disease of anthurium (*Anthurium andreanum* Hort.) (European and Mediterranean Plant Protection Organization, 2009; Chabirand et al., 2014). This disease is prevalent in commercial anthurium nurseries throughout the world and has caused extensive losses to anthurium industries (European and Mediterranean Plant Protection Organization, 2009). Cultural measures (Norman and Alvarez, 1996; Anaïs et al., 2000; Tsang et al., 2010) and chemical bactericides (Nishijima and Chun, 1991) are the most common techniques in blight management. Biocontrol agents (Fukui et al., 1999a, 1999b; Li et al., 2012; Alvarez et al., 2013) have also been adopted as a sustainable approach. However, they are not sufficient to meet the demands on effectiveness, cost and security. It has been widely accepted that developing and growing resistant anthurium cultivars is the most effective and efficient way to control the blight disease (Fukui et al., 1998; Anaïs et al., 2000; Norman et al., 1999; Elibox and Umaharan, 2007).

Screening for resistance to blight caused by *Xad* has been an essential preliminary work of cultivar development. As a result, screening techniques performed on living plants in the glasshouse (Fukui et al., 1998; Norman et al., 1999) and detached leaf discs in the laboratory (Elibox and Umaharan, 2008, 2010) have been developed for the assessment of resistance to bacterial blight in anthurium germplasms. However, because many of the commercial cultivars developed for the anthurium industry were bred mainly for the desirable horticultural traits such as colour, shape, and yield with little attention to blight resistance, not many resistant genetic resources had been identified and applied. Previous results from some cultivars indicated that natural genetic immunity is almost absent, resistance is scarce, and most of them were susceptible to blight in varying degrees (Fukui et al., 1998; Norman et al., 1999; Elibox and Umaharan, 2008, 2010). So, more cultivars and wild accessions should be screened to reveal novel resistance traits.

Plants defend themselves against a pathogen challenge by enhancing the production of reactive oxygen species (ROS), which control many different processes in plants. However, ROS are also toxic molecules and capable of injuring plant cells; for example, high ROS levels can lead to detrimental effects such as damage to lipids, proteins, and DNA (Torres, 2010; Mignolet-Spruyt et al., 2016; Mittler, 2017). Plants have also evolved complex antioxidant defence systems that respond to stresses and mitigate the deleterious effects of ROS (Wrzaczek et al., 2013; Baxter et al., 2014; Noctor et al., 2016; Qi et al., 2017). The major ROS-scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD). In general, the coordination among ROS-scavenging enzymes plays an essential role in ROS levels and the extent of oxidative damage. In addition, other known defence enzymes, such as polyphenol oxidase (PPO, a key enzyme in the synthesis of phenolic compounds with antimicrobial activity) and phenylalanine ammonia-lyase (PAL, the first enzyme in the phenylpropanoid pathway which has important functions in plants following exposure to environmental stresses and pathogen attack), are responsible for defending against pathogens in plants. The activation speeds and activity levels of defence enzymes vary in different plant genotypes or plant-pathogen interactions, so the changes in the levels of these enzymes could serve as valuable physiological indices for the selection of resistant germplasms.

In the present study, we assessed BB resistance in 21 distinct anthurium cultivars which had been released by commercial companies and widely grown in nurseries, by artificially inoculating their leaves with *Xad* cells under laboratory and shaded field conditions. Then the changes in the activities of defence enzymes under blight stress were determined in 6 selected cultivars with different degrees of resistance. The results will facilitate the phytopathological research and resistance breeding in anthurium.

MATERIAL AND METHODS

Bacterial strain and culture conditions

The A6 strain of *Xad* bacteria used in this study was isolated from the commercial *A. andreanum* cv. Arizona grown in a nursery in Hainan Danzhou, and was accurately identified by Gram staining, biochemical tests, pathogenicity tests, specific SCAR-PCR (according to Robène-Soustrade et al., 2006) and 16s rDNA sequencing (Weisburg et al., 1991). The strain was stored in liquid YPGA medium (1 litre containing 7 g yeast extract, 7 g peptone, 7 g glucose; pH 7.2) amended with glycerol (20%) for long-term storage at -80°C prior to use. For inoculation, bacterial cells were routinely cultivated

in liquid YPGA medium for 24 h at 28°C with agitation, and harvested by centrifugation at 8000 g for 5 min. The obtained pellet was resuspended in sterile distilled water and cell populations were adjusted to 3×10^8 colony forming units (CFU) ml^{-1}.

Cultivar resistance evaluation by challenge inoculations and disease scoring

Twenty-one anthurium cultivars (Tab. 1) were propagated by tissue culture in our laboratory, potted in a cinder medium and watered every other day. Plants that were 2.5 to 3 years old were used in the evaluation of resistance to foliar blight in two experiments. For shaded field screening, a special shade house located well away from the breeding and growing nursery was used; plants were brought into the shade house 2 weeks before inoculation in order to acclimatize them to the growing conditions during the wet season; day and night temperatures were maintained at 30 to 37°C and 24 to 28°C, respectively. Inoculations were carried according to Lipp et al. (1992): the second youngest leaves (fully opened but immature) were inoculated with *Xad* A6 suspension at the concentration of 3×10^8 CFU ml^{-1} by injection, with sterile water as the negative control. Following inoculation, the inoculated leaves were covered with transparent plastic bags to maintain humidity for 48 h. At least 12 repeated inoculations were performed on each cultivar. For laboratory screening, the plant material was propagated and grown as above, and transferred into illuminated incubators at one month before inoculation, then grown under a 12 h-photoperiod at 26°C (85% relative humidity), and the inoculation was carried out as previously described. Both experiments were repeated once.

After inoculation, the development of blight symptoms was visually observed and the size of the lesions (spots) was measured every four days; the disease score at 4 weeks was considered the final rating for further analysis as most cultivars had developed bacterial disease symptoms by that time relative to previous observations. Foliage infection was scored based on the following criteria: 0, disease symptoms were absent; 1, spot size < 0.5 cm; 3, spot size 0.5-1.5 cm; 5, spot size 1.6-3.5 cm; 7, spot size

Table 1. Summary of disease response data for *Xad*-infected leaves of 21 anthurium cultivars

No.	Cultivar	Types*	DSI		mean DSI**	RDSI	RRI	Resistance***
			Field	Lab				
1	Tropical	cut	46.9	48.4	47.7a	100.0	0	HS
2	Sharade	pot	39.7	41.3	40.5a	85.0	0.15	HS
3	Sierra	pot	37.4	35.2	36.3a	76.2	0.24	HS
4	Choco	cut	32.1	37.8	35.0a	73.3	0.27	HS
5	Fiesta	pot	30.0	31.4	30.7 bc	64.4	0.36	HS
6	Sweet dream	pot	25.5	28.6	27.1 bcd	56.8	0.43	S
7	Arab	pot	23.0	27.0	25.0 bcde	52.5	0.48	S
8	Cheers	cut	23.2	23.8	23.5 bcde	49.3	0.51	MS
9	Simba	pot	22.2	23.8	23.0 bcde	48.3	0.52	MS
10	Alabama	pot	23.8	21.4	22.6 bcde	47.4	0.53	MS
11	Impreza	pot	17.5	25.4	21.5cdef	45.0	0.55	MS
12	Cherry Red	pot	18.7	21.9	20.3 def	42.6	0.57	MS
13	Arizona	pot	19.0	20.6	19.8 def	41.6	0.58	MS
14	Stallis	pot	20.6	17.5	19.1 def	40.0	0.60	MR
15	White Champion	pot	16.7	15.9	16.3 ef	34.2	0.65	MR
16	Vitara	pot	13.5	18.3	15.9 ef	33.4	0.66	MR
17	New Pink Champion	pot	15.9	15.1	15.5 ef	32.5	0.67	MR
18	Altimo	pot	15.1	14.3	14.7ef	30.8	0.69	MR
19	Red Champion	pot	14.9	12.7	13.5ef	28.3	0.72	MR
20	Manaka	pot	11.3	11.8	11.6 f	24.2	0.76	HR
21	Pink Champion	pot	10.3	9.5	9.9 f	20.8	0.79	HR

*Anthurium varieties for use as pot plants or cut flowers. **Means followed by different letters are significantly different ($p < 0.05$) ($F = 12.94$, $p > F < 0.0001$) according to Duncan's multiple range test. ***HR, highly resistant ($0.75 < RRI < 1$); MR, moderately resistant ($0.6 < RRI \leq 0.75$); MS, moderately susceptible ($0.5 < RRI \leq 0.6$); S, susceptible ($0.4 < RRI \leq 0.5$); HS, highly susceptible ($RRI \leq 0.4$)

> 3.5 cm, extensive necrosis, the infected leaves had died or were about to die. Resistance levels were assessed using the disease severity index (DSI) and relative resistance index (RRI) calculated using the following formulas:

$$DSI = \frac{\sum_{i=0}^{7} (Xi \times Si)}{N \times Smax} \times 100$$

$$RRI = 1 - \frac{DSI}{DSImax}$$

where: i = 0, 1, 3, 5, 7; S_i = disease severity score corresponding to i (here, S_i = i); X_i = number of inoculations given the score of S_i; N = total number of inoculations in a designated cultivar; S_{max} = the highest disease severity score for a designated cultivar; DSI_{max} = the maximum DSI among all the tested cultivars.

Genotypic response across the anthurium cultivars was ranked based on RRI as follows: immune (I, RRI = 1), highly resistant (HR, 0.75 < RRI < 1), moderately resistant (MR, 0.6 < RRI ≤ 0.75), moderately susceptible (MS, 0.5 < RRI ≤ 0.6), susceptible (S, 0.4 < RRI ≤ 0.5), highly susceptible (HS, RRI ≤ 0.4).

Measurement of enzyme activities

In order to determine the nature of antioxidant and defence responses to disease stresses during *Xad* infection, and to assess the valuable physiological indices for the prediction of BB resistance, six cultivars (Pink Champion, Red Champion, Cherry Red, Simba, Sierra and Tropical) with different blight resistance were selected and analyzed for the post-infection enzymatic activity of SOD, APX, CAT, POD, PAL and PPO. The plant material was prepared as that in BB screening in the laboratory, and the second youngest leaves were inoculated with 3 × 10^8 CFU/ml *Xad* according to Lipp et al. (1992), and sampled at 0, 3, 6, 9, 12, 15, 18 days post-inoculation (dpi). Analyses of the SOD, APX, CAT, POD, PAL and PPO activities were carried out according to the methodologies of Beauchamp and Fridovich (1971), Nakano and Asada (1981), Aebi (1984), Maehly (1954), Zucker (1965), and Benjamin and Montgomery (1973), respectively.

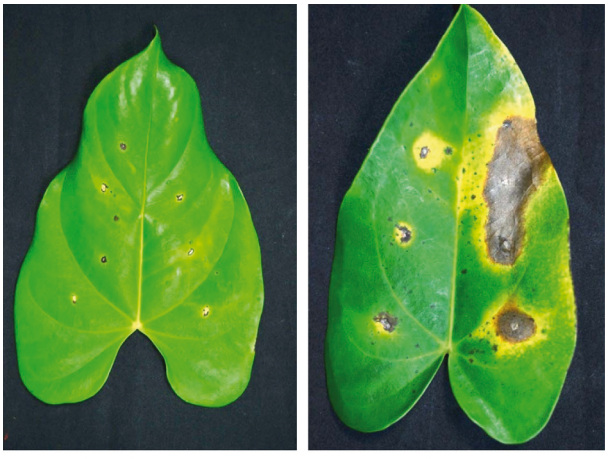
Statistical analysis

The disease severity scores for each assay/cultivar were assessed by using the analysis of variance (ANOVA). DSI and RRI were calculated based on the above equations in Microsoft Excel 2007. The correlation between field and laboratory results was assessed by the determination of the Spearman's rank correlation coefficient (SPSS 21.0 software). The means were compared by Duncan's Multiple Range Test (DMRT) at 5% in SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Each enzyme activity was analyzed with at least three replicates, and the standard deviation (SD) was calculated. Pearson's correlation analyses of RRI and enzyme activities in different cultivars were carried out using SPSS 13.0 software.

RESULTS

Disease resistance of anthurium cultivars

The development of bacterial blight disease occurred non-synchronously in the cultivars; symptoms in the form of water-soaked spots appeared rapidly on the cultivars Sierra and Sharade at 4 dpi and tended to get progressively worse. On the other hand, 'Pink Champion' required a latent period of at least 18 days, while no typical water-soaked spots appeared on 'Manaka' at the final stage of 30 dpi. The DSI data from the shaded field highly correlated with the laboratory results (Spearman's rank correlation coefficient $r = 0.923$, $p < 0.001$), but there was significant genotypic variability among the cultivars (mean DSI ranged from 10.3 to 46.9 in shaded field, 9.5 to 48.4 in laboratory). The cultivar Tropical showed the highest mean DSI (47.7) and was used to determine the comparative RRI values of the remaining cultivars. The RRI data analysis indicated that none of the evaluated cultivars was immune to *Xad*; the cultivars Pink Champion and Manaka were the most resistant (RRI: 0.79 and 0.76,



Pink Champion (Highly Resistant)

Sierra (Highly Susceptible)

Figure 1. Phenotypic responses to *Xad* (3 × 108 CFU/ml) of the anthurium cultivars Pink Champion (left, highly resistant) and Sierra (right, highly susceptible). Foliage infection was scored and photographed 4 weeks after inoculation

respectively), while 'Tropical', 'Sharade', 'Sierra', 'Choco' and 'Fiesta' were the most susceptible (RRI: 0, 0.15, 0.24, 0.27 and 0.36, respectively); the other cultivars responded as moderately resistant (6), moderately susceptible (6), and susceptible (2) (Tab. 1, Fig. 1).

Activities of antioxidant enzymes

According to the results, all the activities of the six enzymes exhibited a drastic increase and peaked (1.28- to 4.22-fold) at 3-15 dpi, indicating that enzyme accumulation was remarkably responsive and sensitive to *Xad* infection. In general, except for PPO, the increment was higher in the resistant cultivar (Pink Champion) than that in the susceptible cultivars (Fig. 2).

Without *Xad* inoculation, all samples of the six cultivars exhibited basal SOD activity (at 0 dpi), with no significant difference among them. After

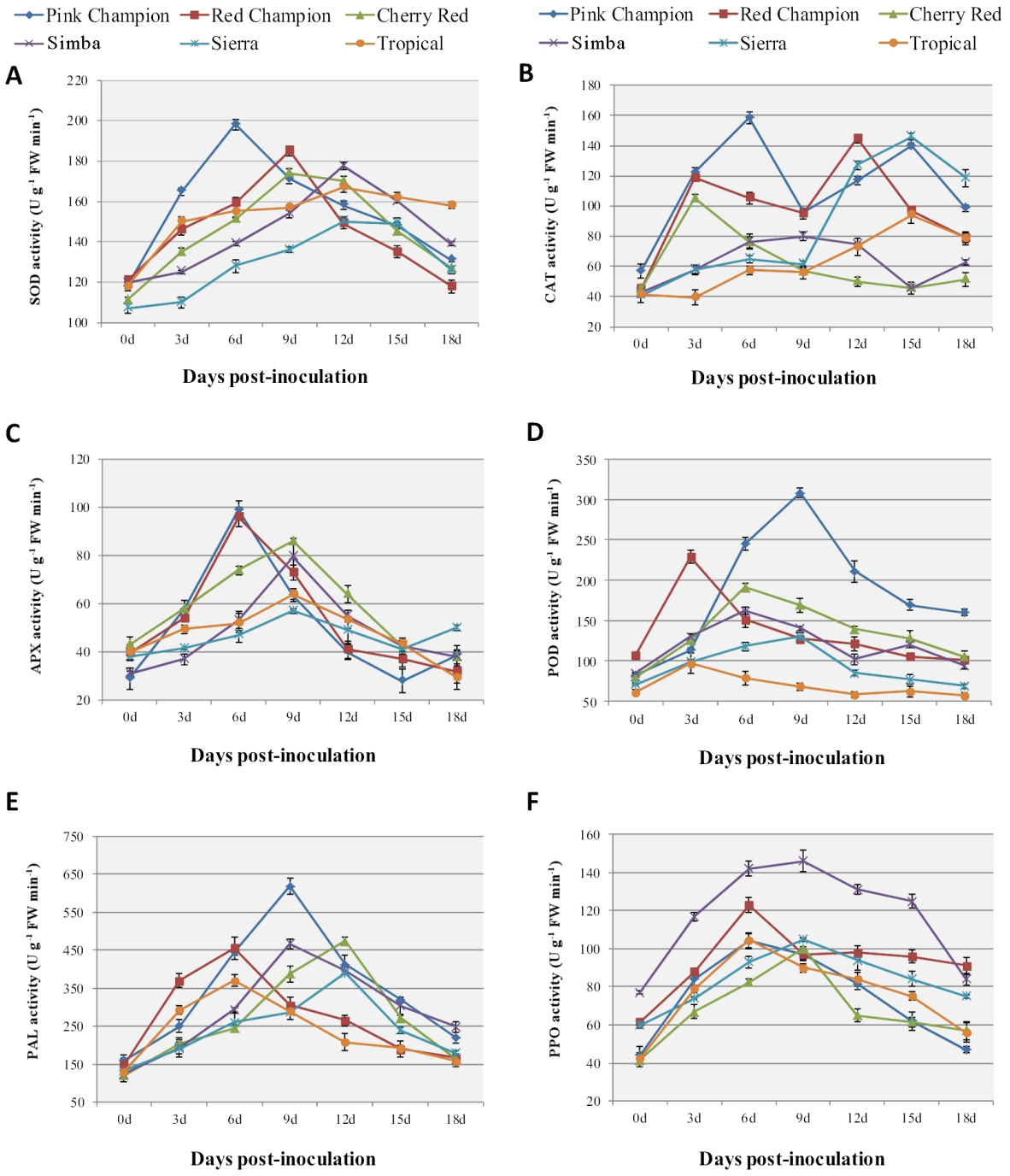


Figure 2. Effect of *Xad* treatment (3×10^8 CFU/ml) on the activities of defence enzymes in the leaves of anthurium cultivars. A) SOD, B) CAT, C) APX, D) POD, E) PAL and F) PPO. Short bars represent standard errors of three replicates

Table 2. Correlation between cultivar resistance and the peak activity of defence enzymes

	RRI	Activity of enzyme					
		SOD	CAT	APX	POD	PAL	PPO
SOD	0.921** (0.009)	1					
CAT	0.323 (0.532)	0.227 (0.666)	1				
APX	0.921** (0.009)	0.968** (0.002)	0.179 (0.735)	1			
POD	0.917* (0.010)	0.930** (0.007)	0.558 (0.250)	0.901* (0.014)	1		
PAL	0.833* (0.039)	0.904* (0.013)	0.464 (0.353)	0.816* (0.048)	0.938** (0.006)	1	
PPO	0.217 (0.679)	0.228 (0.664)	-0.543 (0.266)	0.142 (0.789)	-0.048 (0.928)	-0.014 (0.979)	1

** and *: correlation is significant at the 0.01 and 0.05 level (2-tailed), respectively

inoculation, all the cultivars showed increased activity of SOD with similar changes over time, but the activity of the resistant 'Pink Champion' increased 1.68-fold and reached its peak at 6 dpi, showing a slightly higher peak value and a shorter peak arrival time relative to the SOD activities of the other cultivars (1.4- to 1.56-fold increments, peak time ranging from 9 to 15 dpi, Fig. 2A). CAT activity was also up-regulated in the cultivars after *Xad* inoculation. The resistant 'Pink Champion' and 'Red Champion' exhibited a higher peak CAT activity and a shorter peak arrival time; however, both cultivars showed a character of double peak curves (Fig. 2B). Similar to the patterns of SOD and CAT activities, the APX increment was higher and sooner in 'Pink Champion' (3.41-fold, at 6 dpi) and 'Red Champion' (2.43-fold, at 6 dpi) than in the susceptible cultivars (Fig. 2C). The obtained POD and PAL activities exhibited increasing rates at a shorter peak arrival time in 'Red Champion' (3 dpi and 6 dpi for POD and PAL, respectively) compared to 'Pink Champion'; however, both POD and PAL activities were higher in 'Pink Champion', but peaked a little later, at 9 dpi for both enzymes. For the PPO activity, the six tested cultivars exhibited roughly equal increasing rates accompanying the disease development, which increased 1.75- to 2.41 -fold at peak levels. Somewhat unexpectedly, the MS cultivar Simba had the highest basal PPO activity (at 0 dpi) and the highest blight-induced activity at most time points (3, 6, 9, 12, 15 dpi) compared with the other cultivars (Fig. 2F).

Correlation between cultivar resistance and enzyme activity

According to the results of Pearson's correlation analysis, the relative resistance levels were significantly positively correlated with the activities of the SOD, APX, POD and PAL enzymes in the tested cultivars, with high correlation coefficients (r) of 0.921**, 0.921**, 0.917* and 0.833*, respectively [**p = 0.01; *p = 0.05 (2-tailed); Tab. 2]. However, there was no significant correlation between RRI and the activities of CAT and PPO (r = 0.323, 0.217, respectively). Moreover, the analysis also revealed significant positive correlations among the antioxidant enzymes SOD, APX, POD and PAL.

DISCUSSION

It is generally accepted that the most effective and reliable methods for screening germplasms are under natural infections or on inoculated test plots under field conditions. However, this requires a special site where blight epidemics occur reliably year after year. Another challenge to be considered is that systemic infections occur at the end of a field epidemic. Several studies have reported that systemic and foliage susceptibilities to *Xad* are not well defined (Fukui et al., 1998; Norman et al., 1999; Elibox and Umaharan, 2007). Therefore, it is necessary to perform screening assays which will assess systemic and foliage resistance separately. Typically, field results are also influenced by many factors, such as plant age, plant nutrition, day length, temperature, and others. Plant responses in controlled environments (such as growth chamber) relate to field plant responses, so laboratory screenings that rapidly and accurately assess resistance, particularly the effects of partial resistance components, could be useful and time-saving if they accurately predict field responses.

In this study, a range of anthurium cultivars currently popular on the market were evaluated comparatively in both shaded field and laboratory conditions for blight disease resistance in order to identify potentially useful blight tolerance donors for breeding. Significant differences in the lesion/disease scores among the cultivars, ranging from 1 (highly resistant) to 7 (highly susceptible), were observed under both laboratory and field conditions. Although the ranking of a few cultivars

may have varied between the screening assays (Field vs. Lab), no significant difference in disease severity was observed between the two evaluations (Tab. 1). Under both conditions, the mean DSI of resistance evaluations classified 'Pink Champion' and 'Manaka' as highly resistant genotypes, while 'Tropical', 'Sharade', 'Sierra', 'Choco' and 'Fiesta' were the most susceptible cultivars (Tab. 1), with characteristics similar to those observed in commercial growing surveys. The consistent results suggest that the laboratory screening assay is efficient for reliable and economical identification of the field responses of cultivars to the bacterial blight disease. Moreover, the identified resistant cultivars will be useful as potential sources of resistance for future anthurium breeding programmes.

Oxidative stress is a common secondary stress occurring under biotic and abiotic stresses in plants. To respond to various stresses and mitigate the deleterious effects of ROS, plants have developed complex antioxidant defence systems that include the antioxidant enzymes SOD, APX, CAT, POD and others (Inupakutika et al., 2016; Noctor et al., 2016; Mittler, 2017). In this study, although the activities of antioxidant enzymes increased steadily under blight stress in all the cultivars, with similar change tendencies over time, the accumulation rates and activity levels were markedly higher in the resistant cultivars than in the susceptible ones. This suggests that the antioxidant enzymes appeared to eliminate ROS more efficiently so that cells were protected against oxidative damage in the resistant cultivars. Two peaks of CAT generation were observed in the highly resistant 'Pink Champion'(at 6 and 15 dpi) and in the resistant 'Red Champion' (at 3 and 12 dpi), with both activities undergoing a distinct decline at 9 dpi, but remaining still higher relative to the other susceptible cultivars at the same time, which exhibited only a single peak after inoculation. This might be due to differential protection mechanisms operating in resistant cultivars whereby they maintain higher CAT activities for many more days after infection with *Xad*. PAL is the first enzyme in the phenylpropanoid metabolism pathway, responsible for the biosynthesis of *p*-coumaric acid derivatives, phytoalexin, salicylic acid, and lignins that contribute to plant defence systems. Similar to the antioxidant enzymes, PAL accumulated quickly at an early stage in the resistant cultivars, where it reached a peak at 6 dpi ('Red Champion') and 9 dpi ('Pink Champion'), and increased 3.07- and 3.86-fold, respectively. The correlation analysis also indicated that PAL levels were closely related to blight resistance in anthurium ($r = 0.833$, Tab. 2). This result is in agreement with previous findings that PAL was involved in increasing resistance and its activity significantly increased in response to plant pathogens (Droby et al., 2002; Ballester et al., 2010; Kim and Hwang, 2014; Tonnessen et al., 2015). PPO can catalyze the formation of lignin and antimicrobial phenolic substances through oxidizing phenolic compounds; its rapid accumulation is important for resistant genotypes following infection (Tyagi et al., 2000; Mayer, 2006). Unexpectedly, however, the MS cultivar 'Simba' showed the highest basal and blight-induced peak PPO activity, while for the rest of the cultivars, the accumulation rates and maximum PPO activities were roughly equal, no matter how resistant or susceptible they were. This result demonstrated that there was no significant correlation ($r = 0.217$) between the changes in PPO and the variation in resistance to the bacterial blight disease in anthurium.

CONCLUSIONS

1. By screening a set of commercial cultivars under field and laboratory conditions, we identified anthurium cultivars that performed well under bacterial blight stress, which allows them to be recommended for use in resistance breeding.
2. The results of this study also refer to the activities of defence-related enzymes. All the cultivars tested showed induced alterations in enzyme activities upon *Xad* challenge, but only the resistant cultivars exhibited a more rapid accumulation and higher activities of these enzymes.
3. SOD, APX, POD, and PAL activities were closely related to the resistance of anthurium cultivars to foliar blight; these biochemical markers would be a useful tool for the early identification of blight resistant anthurium clones.

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AUTHOR CONTRIBUTIONS

J.H.N, Y.C. and Y.M.Y. – carried out the experimental work; J.J.N. and J.M.Y. – designed the experiment, supervised the laboratory work, and edited the

manuscript; J.J.N, X.E.L. and Q.Y.L. – contributed to manuscript writing and performed the statistical analysis of the data.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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