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## Accepted Manuscript

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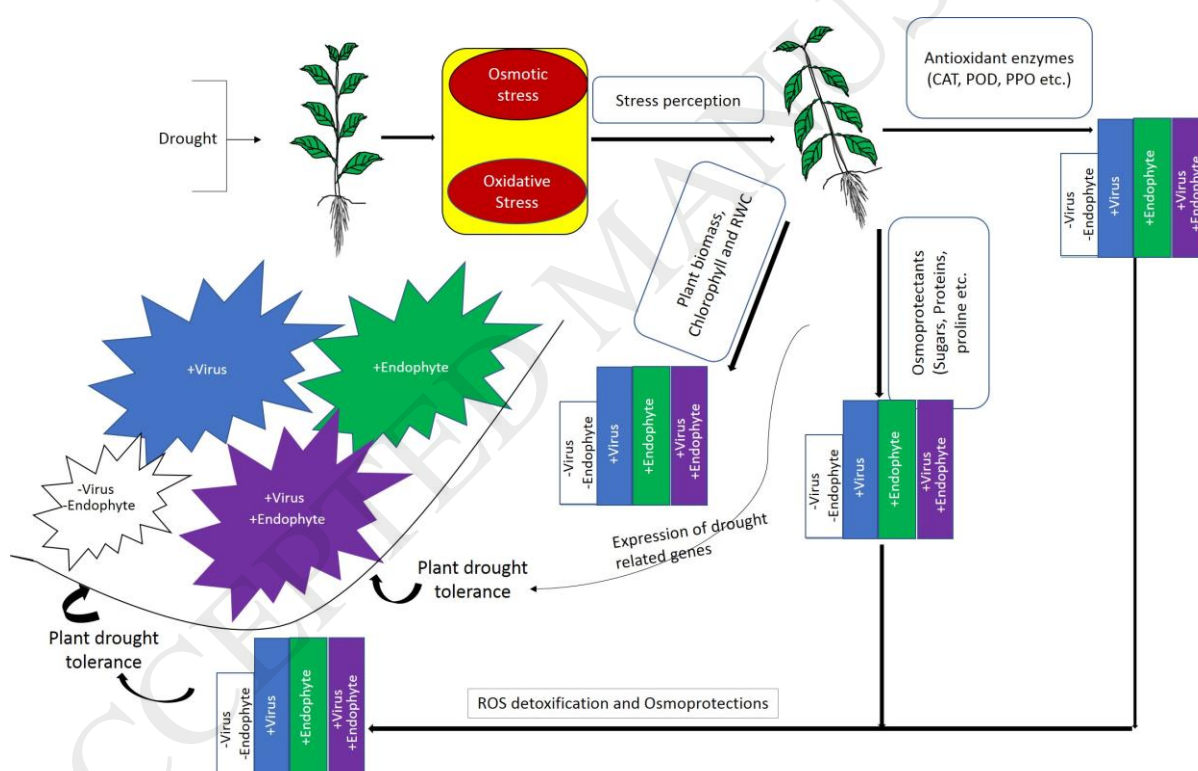
## Fungal endophytes and a virus confer drought tolerance to *Nicotiana benthamiana* plants through modulating osmolytes, antioxidant enzymes and expression of host drought responsive genes

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### Graphical abstract



### Highlights

- Water stress tolerance of endophyte-colonized plants was correlated with increases in plant biomass, RWC, osmolytes, and antioxidant enzymes.
- There was significant upregulation of drought-related genes in endophyte colonized plants.

- Both fungi and the virus reprogram plant responses to water stress in a similar way.
- Co-infection of fungi and virus neither had an additive nor a regressive effect on plant drought responses.

## Abstract

Microbial symbionts increase plant growth and eco-physiological performance under abiotic stress. In this study, we evaluated how the colonization of two fungal endophytes isolated from wild *Nicotiana* species from areas of drought-prone northern Australia, and a plant virus, yellowtail flower mild mottle virus (genus *Tobamovirus*), improved water stress tolerance in *N. benthamiana* plants. Inoculation with both of the two fungal strains used and the virus significantly increased plants tolerance to water stress as manifested by their significant delay in wilting of shoot tips. The water stress tolerance of fungus-inoculated plants was correlated with increases in plant biomass, relative water content, soluble sugar, soluble protein, proline content, increased activities of the antioxidant enzymes catalase, peroxidase and polyphenol oxidase, decreased production of reactive oxygen species, and decreased electrical conductivity. In addition, there was significant upregulation of several genes previously identified as drought induced. The influence of the virus was similar to the fungi in terms of increasing the plant osmolytes, antioxidant enzyme activity and gene expression. Although separate infection of fungi and virus increased plant water stress tolerance responses, their co-infection in plants did not have an additive effect on water stress responses. These findings show that both fungi and viruses reprogram plant responses to water stress in a similar way.

**Keywords:** Antioxidants; chlorophyll; drought tolerance; gene expression; fungi; osmolytes; plant biomass; ROS; wilting.

## 1. Introduction

Plants, being sessile, are continuously challenged with environmental stimuli and stresses, which significantly deter their growth and survival. Of these, water deficit or drought stress is one of the most important limiting factors for the growth of plants in both natural and agricultural settings (Passioura, 2006). Current climate change models are predicting a substantial decrease in soil water availability for plants in the coming years (IPCC, 2015).

The way plants respond to water stress is rather complex and involves various physiological and morphological adaptations (Chaves et al., 2009; Flexas et al., 2006; Nobel, 1999). Water deprivation

causes an imbalance in the osmotic potential of the plant tissues, and induces the synthesis of reactive oxygen species (ROS) (Jakab et al., 2005). Plants accumulate compatible solutes or osmolytes such as sugars, proteins, proline, etc. to maintain their cellular redox potential and osmoregulation (Dar et al., 2016; Joshi et al., 2016; Lisar et al., 2012; Munns, 1988; Serraj and Sinclair, 2002a; Slama et al., 2015). The plant also produces reduced glutathione (GSH), polyphenols, and several antioxidant enzymes like catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), etc. to counter the detrimental effects of increased ROS (Claeys and Inzé, 2013; Hoekstra et al., 2001). Many drought-related genes have been identified (Seki et al., 2002) that can be grouped into two major classes: i) proteins that function directly in abiotic stress tolerance, and ii) regulatory proteins, which are involved in signal transduction or expression of stress-responsive genes (Shinozaki et al., 2003).

Several microorganisms such as bacteria and fungi have been found to increase plant tolerance to water stress. Fungal induced plant water stress tolerance has been reported for both mycorrhizae (reviewed by Rapparini and Peñuelas 2014) and endophytes (reviewed by Dastogeer and Wylie, 2017; Rodriguez et al. 2009). In particular, fungal endophytes have been shown to increase drought-tolerance in many crops through morphological and biochemical mechanisms such as increased photosynthetic rate and water use efficiency (Rozpadek et al., 2015; Swarthout et al., 2009), higher accumulation of osmoprotectants or compatible solutes (Grover et al., 2011), improving the plant nutrient availability and root growth (Malinowski and Belesky, 2000b) and regulation of genes implicated in homeostasis (Estrada et al., 2013).

The study of plant viruses has been biased towards investigating pathogens. The study on the positive role of virus induced plant stress tolerance is, however, limited. Several viruses provide benefits to their hosts undergoing stress. For instance, a three-way mutualism between a virus, a fungus and a plant conferred thermal tolerance (Márquez et al., 2007). Infection with some RNA plant viruses such as brome mosaic virus (BMV), cucumber mosaic virus (CMV), tobacco mosaic virus and tobacco rattle virus increase drought tolerance through the accumulation of osmoprotectants and antioxidants (Xu et al., 2008). Plant tissue contains various RNA viruses, but their roles in plant ecophysiology to a large extent are still unstudied. Because plants simultaneously interact with biotic and abiotic agents, biotic and abiotic signalling pathways share common nodes and their output has substantial overlaps. This may be why infection with some microbes primes plants to survive under complex environmental conditions (Chini et al., 2004; Timmusk and Wagner, 1999; Xu et al., 2008).

We aimed to study the effects of infection by two ascomycete fungal endophytes and yellow tail flower mild mottle virus that were identified previously (Dastogeer et al., 2017a). *N. benthamiana* is an important model plant in plant virology and other branches of plant science (Goodin et al., 2008). Here we described the changes in plant biomass, relative water content, chlorophyll content, electrolyte leakage, accumulation of osmolytes and some antioxidant enzymes as well as expression of drought

related genes in *N. benthamiana* plants caused by fungal and virus infection and drought stress and by their interactions.

## 2. Materials and methods

### 2.1 Endophyte strain, virus and plant materials

Two ascomycete endophytic fungal strains; E-162 (*Cladosporium cladosporioides*, GenBank accession: KU059880) and E-284, (unidentified member of the Ascomycota, GenBank accession: KU059897) were previously isolated from sterilised root tissues of native *Nicotiana benthamiana* plants (Dastogeer et al., 2017c). These isolates were chosen based on their positive influence on water stress tolerance of *N. benthamiana* plants (Dastogeer et al., 2017a). We used yellow tailflower mild mottle virus isolate Cervantes (YTMMV, genus *Tobamovirus*, GenBank accession KF495565) which was originally collected from a wild plant of Yellow Tailflower (*Anthocercis littoria*, family Solanaceae) at Cervantes, Western Australia (Wylie et al., 2014). For the current study, we chose an accession of wild *N. benthamiana* plant, Mta5, originally isolated from an Arid region near Mt Augustus, Western Australia. This accession was identified to have the highest inherent tolerance to water stress from a number of accessions of Australian *Nicotiana* species tested (unpublished). This virus and the plant accession were chosen because this virus was not lethal to the wild accessions of *N. benthamiana* plants we used. Instead, it results in systemic spread within plants as was confirmed by RT-PCR, and produces moderate symptoms of chlorosis, leaf mosaic and deformation. It causes moderate stunting of growth and reduces flower production (Wylie et al., 2015).

### 2.2 Plant growth conditions and inoculation

Surface-sterilised seeds were prepared by treatment with 3% sodium hypochlorite for 3 min and then 75% ethanol for 2 min, then rinsed with sterile water three times. Three weeks after sowing, seedlings were uprooted and roots were washed in running water. Before transplanting each seedling in an individual pot filled with sterilised soil, the roots were inoculated with fungal mycelium. For inoculum preparation, fungal strains were grown on potato dextrose broth (0.1X) at 25°C for 7 d. The mycelia were collected, and the mycelial suspension was prepared in a liquid homogenizer-mixer by maceration. The large hyphal bits were removed by filtration through sterile absorbent cotton wool plugs. With the help of a haemocytometer and a compound microscope, the inoculum concentration was counted and adjusted to the desired level ( $5 \times 10^4$  fragments mL<sup>-1</sup>) through sterile dilution (Dastogeer et al., 2017a). The viability of mycelial fragments was assessed by a germination test on PDA after incubation for 48 h at 25°C.

Inoculation of *N. benthamiana* seedling was done by dipping the roots in the inoculum suspension for 5 h before planting. The control seedlings were mock inoculated with sterile distilled water. The seedlings then were transplanted in steam-treated soil in pots with one seedling per pot (10 cm in diameter and 12 cm deep, 800 g soil per pot). Steam treatment of soil was done at 99°C for 4 h twice

with a gap of 48 h in between steaming sessions to eliminate other microbial contaminants. Sterilised media was used to observe the effects only of these strains in the absence of other fungi. Inoculation efficiency was achieved by applying the inoculum suspension by spraying at the root-zone of the seedling at the time of planting. The plants were grown in an insect proof greenhouse at 22°C days and 17°C night, 60±5% RH, with a natural photoperiod. They were watered regularly and received weekly nutrient feeds.

After two weeks of fungal inoculation, half of each group was subjected to mock inoculation with 0.1M phosphate buffer (pH 7) and diatomaceous earth (Sigma) and another half as above with the addition of macerated leaf material from YTMMV infected plants (Wylie et al., 2015). After two weeks when the YTMMV inoculated plants showed mild mosaic symptoms on the leaves, water stress was applied to half of each group by withholding watering. Plants were grown under the condition described above except that the water stress group did not receive watering. The fungal infection status was checked by culturing surface-sterilized roots and identifying the isolated fungi by sequencing of the ITS region of the rDNA (Dastogeer et al., 2017c).

### **2.3 Biomass and relative water content (RWC) measurement**

Biomass is an overall measure of plant fitness. For the measurement of biomass, six plants from each treatment combination (watered vs. stressed; YTMMV vs. mock inoculated; E-162 vs. E-284 vs. non-inoculated, NE) were removed from the potting medium at 11 d after water stress application. The roots were washed clean, and the plants were cut at the crown to separate root and shoot. Biomass dry weight was measured after oven drying the samples at 75°C for 48 h.

To measure RWC, fully expanded leaves from twelve plants from each treatment combinations were sampled at d11 after water stress treatments. The leaves were weighed just after sampling to obtain fresh weight ( $W_f$ ) and were submerged in tubes containing distilled water. The tubes were then stored at 4°C in darkness for 24 h to achieve full turgidity of the leaves. Then, the leaves were removed from the tubes, and the turgid weight ( $W_t$ ) was determined. Finally, the leaves were oven-dried (48 h at 75°C) to obtain dry weight ( $W_d$ ). The leaf relative water content was determined as  $RWC = [(W_f - W_d) / (W_t - W_d)]$  (Barrs and Weatherley, 1962)

### **2.4 Determination of electrolyte conductivity (EC) and chlorophyll content (ChlC)**

We followed the techniques of Sullivan (1971) with modifications to determine the membrane stability of leaves. Fully expanded leaves were collected from plants at 0, 4, 8 and 11 days after stress treatment. Leaves were washed with deionized water to remove leaf surface-adhered electrolytes. To allow electrolyte diffusion from the leaf tissue, leaf discs (5 mm diameter) were placed in a test tube containing 10 ml of deionized water and incubated at 25°C for 4 h. After incubation, tubes were

brought to 25°C, shaken, and the initial conductance (EC1) was determined with a digital electric conductivity meter. Tubes were then placed in an autoclave at 121°C for 20 min to completely kill leaf tissues, releasing all electrolytes. After cooling to 25°C, shaken, and final conductance was remeasured (EC2). There were twelve plants from each treatment combinations (watered plus stressed; YTMV plus mock inoculated; E-162 plus E-284 plus NE inoculated). Leaf electrolyte leakage was calculated as a percentage as follows:  $LEL = EC1/EC2 \times 100$ .

Chlorophyll content of leaves was measured by using a hand-held chlorophyll meter (CCM-200plus, Opti-Science) at 0, 4, 8 and 11 d after stress treatment from six plants of each treatment combination. Chlorophyll measurement was taken during mid-day from three fully expanded leaves from the top, and the readings were averaged.

## 2.5 Determination of sugar, protein and proline

Leaf samples for sugar, protein and proline determination were collected at 0, 4, 8 and 11 d after water stress treatments from six plants per treatment combination. Immediately after harvesting leaves were snap frozen in liquid nitrogen and preserved at -80°C until use.

### *Proline content (μmols/gram).*

Proline was estimated according to the method described by Bates et al. (1973) with minor modifications. Approximately 0.05 g of frozen leaf tissue was homogenised in 5 ml of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 4000 xg for 10 min at 4°C. One millilitre of the supernatant was mixed with 1 ml of acid ninhydrin and 1 ml of glacial acetic acid in a test tube. The mixture was placed in a boiling water bath for 1 h. The reaction mixture was extracted with 3 ml of toluene, and the absorbance was measured at 520 nm with a spectrometer (Lambda 25, PerkinElmer). Appropriate proline standards were used for calculation of proline levels in the samples.

### *Soluble sugar content (mg/g)*

Total soluble sugar content was estimated using the method of Dubois et al. (1951). Frozen leaves (0.05 g) of each sample were homogenised in 5 ml of 80% ethanol and incubated for 1 h at 80°C in water bath. In 0.5 ml extracts, 0.5 ml distilled water and 1 ml of 5% phenol were added and incubated for 1 hr at 25°C. After adding 2.5 ml sulphuric acid in the solution, the absorbance was read at 490 nm with a spectrometer (Lambda 25, PerkinElmer). Glucose was used as a standard.

### *Soluble protein content (mg/g):*

The total soluble protein was estimated by a dye-binding assay as described in Bradford and Williams (1976) with some modification. Leaf materials (0.05 g) were homogenised in 1.5 ml of phosphate buffer solution and centrifuged at 4000 xg for 5 min. Then, 0.1 ml of extract was placed in a test tube containing 5 ml protein reagent. One litre of protein reagent was prepared with 100 mg Coomassie



Brilliant Blue-G250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid in water. The absorbance of samples was read at 595 nm against a blank with a spectrometer (Lambda 25, PerkinElmer). Bovine Serum Albumin (BSA) was used to prepare the standard curve.

## **2.6 Determination of reactive oxygen species (ROS) sensitivity.**

For ROS determination leaves were harvested at 11 d after stress treatment. Using a cork borer, discs (2 mm) from fresh leaves were obtained from each of the treatment combinations and placed in a solution of 100  $\mu$ M of the herbicide paraquat (N,N'-Dimethyl-4,4'-bipyridinium dichloride, Sigma, CAS No.# 856177-) and incubated at 22 °C under fluorescent lights. After 48 hr exposure to paraquat, leaf discs were observed to record chlorophyll oxidation visualised by tissue bleaching (Redman et al., 2011).

## **2.7 Measurement of the antioxidant enzyme activity**

The activity of catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO) were chosen as representative antioxidants to estimate the plant's responses to water stress, endophytes and virus infection. Leaf samples were collected at 0, 4, 8 and 11 d after water stress treatments from twelve plants per treatment combination. Immediately after harvesting, leaves were snap frozen in liquid nitrogen and preserved at -80 °C until use. Plant leaves (100 mg) were homogenised in 5 ml phosphate buffer (0.1M, pH 7.0) and then centrifuged (4000xg rpm for 15 min at 4°C). The supernatant was used for all enzymatic analyses. All parameters were expressed as activity per mg protein. Total proteins were determined according to Bradford method as described above (Bradford and Williams, 1976).

The CAT activity was estimated as described by Aebi (1984). In the 0.5 ml crude enzyme supernatant 0.5 ml phosphate buffer (10 mM, 7.0 pH and 0.5 ml H<sub>2</sub>O<sub>2</sub> (0.2M) were added. After 1 min incubation, the reaction was stopped by adding 4 ml H<sub>2</sub>SO<sub>4</sub> (2%, v/v). The absorbance of the reaction solution was measured at 240 nm. An absorbance change of 1 min was defined as  $\mu$ g of H<sub>2</sub>O<sub>2</sub> released per mg protein per min.

The POD and PPO activities were measured as described by Kar and Mishra (1976) with a little modification. To the 0.1 ml crude enzyme supernatant, 0.4 ml phosphate buffer (0.1 M), 50  $\mu$ l pyrogallol (1 M, Sigma, CAS No.# 254002) and 50  $\mu$ l H<sub>2</sub>O<sub>2</sub> (30%) were added. After incubation (1 min at 25°C), the reaction was stopped by adding 5% (v/v) H<sub>2</sub>SO<sub>4</sub> (4 ml). The amount of purpurogallin synthesised during the reaction was measured by the absorbance at 420 nm. A similar assay mixture (without H<sub>2</sub>O<sub>2</sub>) was prepared for determination of polyphenol oxidase and the absorbance of purpurogallin formed was read at 420 nm after 5 min incubation at 25°C.

## 2.8 Gene expression analysis using RT-qPCR

The effect of fungi and virus infection on relative expression of drought related genes was studied. Leaf samples were collected at 8 d after initiation of water stress and kept under  $-80^{\circ}\text{C}$  until use. Total RNA was extracted using QIAGEN RNeasy plant mini kit per the manufacturer's instructions. The quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis was performed using QIAGEN OneStep RT-PCR kit in Rotor-Gene Q instrument (QIAGEN) and the gene-specific primers described in Table 1. Thermal cycling conditions utilized a reverse transcription step at  $50^{\circ}\text{C}$  for 30 min;  $95^{\circ}\text{C}$  for 15 min (RT inactivation and initial denaturation step), followed by 40 PCR cycles at  $94^{\circ}\text{C}$  for 40 sec,  $60^{\circ}\text{C}$  for 30 sec and 72 for 1 min and final extension at  $72^{\circ}\text{C}$  for 10 min. The full coding sequence of genes of interest was obtained from the NCBI (National Center for Biotechnology Information) database, and primers were designed using primer3 in accordance with the criteria required for quantitative PCR primer design (Udvardi et al., 2008). *Actin* was chosen as the reference gene due to its constitutive and stable expression (Staiger, 2000; Tian et al., 2015a; Vergne et al., 2007). The expression levels of each gene were normalised using *actin* as a reference gene, and relative expression of genes were derived using  $2^{\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001).

## 2.9 Plant wilting behaviour

Three different parameters were considered: days to showing first leaf wilting symptoms; days to final leaf wilting and days to shoot tip wilting (irrecoverable damage) after initiating the water stress. Six plants from each of the treatment combinations (YTMMV vs. Mock; E-162 vs. E-284 vs. NE and all possible combinations) were chosen, and water stress applied. to the plant. This experiment was repeated once again under same experiment condition and experimental design.

## 2.10 Statistical analysis

The three-way ANOVA was performed separately on data under well-watered and stressed conditions. The variables were sugar content, protein content, proline, CAT, POD, PPO, EC, ChlC, while the independent variables were days of water stress, fungal inoculation, and virus inoculation status. For parameters, such as RWC, biomass traits, ROS sensitivity and gene expression analysis the data were obtained only at a single time point, so the independent variable was not present in the three-way ANOVA analysis. Instead, water treatment, fungal inoculation, and virus inoculation were the three independent variables considered. The multiple comparisons among the treatments were computed using Tukey's HSD tests. For analysing the wilting behaviour of the plants in response to water stress, the data from the two separate experiments were pooled because we did not find any significant difference between the experiments. A two-way ANOVA was performed to determine the effect of fungi and virus, and their interaction on plants stress tolerance. The ANOVA and multiple comparison tests were performed in statistical software R using package agricolae (De Mendiburu,

2016). The line graphs, bar charts and box plots were created in Microsoft Excel-2016, and the mean and standard error values were calculated in PAST (Hammer et al., 2008).

### 3. Results

#### 3.1 Plant Biomass, relative water content and chlorophyll content

Water stress significantly affected plant biomass, and it caused a decreased shoot and root dry mass as well as root/shoot ratio (Table 2). The presence of the fungal endophyte did not show any effect on biomass traits under well-watered conditions, but both the fungal strains produced greater root biomass and root/shoot ratio under stress only in the absence of YTMMV (Table 2). It was noted that the effect of the YTMMV infection was significant and it caused lower accumulation of shoot and root biomass under the well-watered condition and a lower accumulation of shoot biomass under stressed conditions irrespective of fungal inoculation (Table 3). Infection of the virus had no apparent effect on root/shoot ratio in any case (Table 2). We did not find any interaction between fungi and virus or three-way interaction of virus, fungus and water stress on plant biomass accumulation (Table 2).

The RWC was dependent on water stress treatment and endophyte status, water stress  $\times$  endophyte status and water stress  $\times$  virus inoculation on plants (Table 2). There was no interaction between endophyte infection status and virus inoculation status and three-way interactions of water stress, endophyte infection and virus inoculation (Table 2). A significantly lower level of water content was measured from all samples subjected to stress (Fig. 1). Non-endophytic samples, however, showed substantially decreased RWC compared to the endophytic plants with either of the strains (E-162 or E-284) regardless of virus inoculation. Virus infection did significantly influence RWC of plants under stress. The YTMMV significantly negatively affected leaf chlorophyll pigment under all conditions (Fig. 2a). Water stress caused a reduction in leaf chlorophyll only at d11 in the absence of virus in NE and E-284 plants but did not affect in E-162 plants (Fig. 2a).

#### 3.2 Reactive Oxygen Species

Excised leaf discs from *N. benthamiana* seedlings grown under well-watered and under stress conditions with or without fungus and/or virus were analysed for ROS sensitivity. The photobleaching of leaf disc by herbicide paraquat is mimicking to ROS sensitivity.

Leaves that were not subjected to water stress remained green indicating that ROS was not produced by exposure to paraquat and the effect of fungi and virus was neutral. When exposed to water stress, plants colonized with either of the fungal strains showed no significantly different in photobleaching than those uncolonized (Table 3) in the absence of virus. Interestingly, under water stress, virus

infected plants showed significantly lower photobleaching than non-infected plants, and there was no interaction effect of the virus and fungal colonization in any condition (Table 3).

### 3.3 Electrical conductivity

Under well-watered conditions, there were no differences in electrolyte conductivity (EC) of leaves among the treatments across sampling points (Fig. 2b). Water stress significantly influenced EC of leaves, and it increased as the stress progressed in all plants. However, the rate of the increase in non-endophytic plants was significantly higher as compared to the endophytic plants at 8 d and 11 d. This increasing trend continued in all groups as the stress progressed in absence of virus. It was noted that the YTMMV virus inoculation increased the EC under water stress at d4 in the non-endophytic plants but not significantly so in endophytic plants. Under well-watered condition, YTMMV increased EC in NE and E-284 plants but not in E-162 plants at d11 (Fig. 2b).

### 3.4 Accumulation of osmolytes

#### *Soluble Sugars:*

None of the endophyte strains had any noticeable influence on the plant soluble sugar levels across sampling points under well-watered conditions. Inoculation with YTMMV seemed to increase the sugar level in endophytic plants to a certain extent under well-watered conditions (Fig. 3a, Table 4).

When drought was applied, sugar levels showed a steady increase in all the groups irrespective of endophyte or virus inoculation status even though there were significant differences in the level of sugar between endophytic and non-endophytic plants (Fig. 3a, Table 4). In contrast, virus inoculation did not influence sugar accumulation in the plant under water stress (Fig. 3a, Table 4).

#### *Soluble protein:*

The amount of soluble protein in leaves showed a sharp increase under water stress conditions regardless of fungal or viral inoculation (Fig. 3b). When there was no virus inoculation, endophyte treatment alone resulted in significantly higher protein accumulation than non-endophytic plants. YTMMV infection resulted in a steeper increase (> four times the level under control condition) in the level of protein in both endophytic and non-endophytic plants (Fig. 3b). The YTMMV inoculation increased soluble proteins in the plants also under the well-watered condition, and no influence of any of the endophyte isolates was observed (Fig. 3b and Table 4).

#### *Proline content:*

Proline levels measured low and were constant in well-watered (Fig. 3c). None of the endophyte strains had any influence on proline in well-watered plants, but inoculation with YTMMV increased proline levels to some extent as the plants grew older (Fig. 3c). The levels of proline increased dramatically in plants challenged with increasing water stress in all treatments (Fig. 3c). There were significant differences in proline levels between endophytic and non-endophytic plants in the absence

of virus inoculation. But this difference was not observed when the plants were inoculated with the YTMMV (Fig. 3c). Therefore, it was noted that virus inoculation had a substantial impact on leaf proline levels in both stressed and well-watered conditions (Fig. 3c).

### 3.6 Changes in antioxidant enzymes

#### *Catalase*

Catalase activity increased under water stress treatment. The rate of increase was highest in E-162 inoculated plants (Fig. 4a). Infection of YTMMV caused a steady increase in CAT activity, which became more prominent as water stress progressed (Fig. 4a). No additive effect of virus and fungal inoculation was noted regardless of water status in the plants except at d8 under stress (Table 4 and Fig. 4a).

#### *Peroxidase*

Water stress caused increased peroxidase activity in plants in all treatments. At severe water stress, the peroxidase activity was significantly higher in endophytic plants with either of the fungal strains (Fig. 4b). The virus inoculation resulted in a higher peroxidase activity regardless of water availability although the increase was much more pronounced under water stress conditions (Fig. 4b). We did not observe any interaction effect of the virus and fungal inoculation on peroxidase activity under any condition except at d11 (Table 4 and Fig. 4b).

#### *Polyphenol oxidase*

Water stress increased polyphenol oxidase activity in all plants. Plants inoculated with E-284 had a significantly higher polyphenol oxidase activity under both conditions in presence of virus (Fig. 4c). Virus inoculated plants had a higher PPO activity under normal watering or stress conditions even though the rate of increase was much more prominent under water stress (Fig. 4c). We did not observe any interaction effect of the virus and fungal inoculation on peroxidase activity under well-watered conditions but did observe the interaction of E-284 and the virus under stress condition (Table 4 and Fig. 4c).

### 3.7 Expression of drought stress related genes

Water stress and virus infection separately caused differential expression of most of the genes under investigation. There was a significant interaction between water stress and YTMMV inoculation on NbCAT3, NbDreb2a, NbGBP16, NbMYB, NbPAL1, and NbSOS1 (Table 5). The NbCAT3 was significantly upregulated (8-11 fold) in YTMMV infected plant under well-watered conditions, which increased even more (17-20 fold) under stress (Fig. 5). The fungus-infected plants did not show any difference in expression compared to non-inoculated plants under well-watered conditions in absence of virus. Under stress however. However, under stress, NbCAT3 expression differed as a function of fungal inoculation (Fig. 5) in absence of the virus. NbDreb2a expression was significantly

downregulated under stress and due to YTMMV inoculation (Fig. 5), and there was a significant interaction of stress and virus infection (Table 5). The expression of NbGBP16 was upregulated under water stress, and E-162 inoculation caused a further increase in its expression in the absence of virus. Interacting with YTMMV, water stress resulted in higher expression (20-24-fold compared to non-inoculated control) regardless of endophyte inoculation. While water stress caused in general a higher expression of NbHSP101 gene its expression was much higher in non-endophytic plants than corresponding endophytic plants (Fig. 5).

There were significant three-way interaction effects of water treatment, endophyte inoculation and virus inoculation as well as all possible three-way interaction effects on the expression of this gene (Table 5). E-162 caused significant upregulation of NbMYB gene under stress but only in the absence of virus. In presence of virus, stress induced NbMYB expression did not differ among endophytic or non-endophytic plants. (Fig. 5). E-162 had a marked influence on NbMYC expression in the absence of YTMMV (Fig. 5). Expression of NbPAL1 was upregulated by virus infection in well-watered condition only (Fig. 5). In absence of virus fungal inoculation increased NbPAL1 expression under stressed and watered conditions. NbSOS1 expression was increased by YTMMV infection in well-watered plants regardless of fungal presence. The gene NbPDH1 and NbWRKY were significantly upregulated in endophytic plants under water stress irrespective of YTMMV infection (Fig. 5). The expression of NbZfP was increased by water treatment and virus infection, but their interaction was not significant (Fig. 5, Table 5) and the role of fungal inoculation was not apparent on its expression (Table 5).

### **3.8 Plant wilting behaviour under water stress:**

Plants respond to water stress by wilting of leaves followed by wilting of the shoot tip and ultimately death at the severe stage. In our experiment, we found that inoculation with YTMMV delayed plant initial responses to stress. Virus-inoculated plants took a significantly longer time to begin leaf wilting irrespective of fungal inoculation. None of the fungal strains had an apparent effect on the plants early response to water stress (Fig. 6). As wilting progressed, virus inoculated plants took significantly more days to fully wilt. Regarding the shoot tip, both fungal strains significantly delayed plant shoot tip wilting in the absence of the virus. For example, plants inoculated with E-162 or E-284 took an average of 19 days for the shoot tip to wilt, whereas non-inoculated plants wilted within 14 dps (days post stress) (Fig. 6). However, in the presence of YTMMV infection, all the plant significantly delayed shoot tip wilting (average: 23-25 dps) compared to virus free plants (14-19 dps) and the effect of fungal infection was not apparent (Fig. 6).

## 4. Discussion

### 4.1 Plant responses to water stress

None of the fungal strains increased plant shoot biomass either under well-watered or stressed conditions. This result is in line with several other studies showing a neutral effect of fungal endophytes on shoot biomass under stress (Briggs et al., 2013; He L, 2017; Hesse et al., 2003, 2005; Hill et al., 1996; Jia et al., 2015; Kane, 2011; Khan et al., 2013; Oberhofer et al., 2014; Rudgers and Swafford, 2009). In contrast, certain endophytic strains were also reported to increase (Ghabooli et al., 2013; Gibert et al., 2012; Hill et al., 1996; Malinowski et al., 2005; Vazquez-de-Aldana et al., 2013; Zhang and Nan, 2007) or decrease (Assuero et al., 2006; He L, 2017; Rahman et al., 2015; Yang et al., 2014; Zhang, 2017) plant shoot biomass under water stress. Previously, we reported increased shoot biomass of *N. benthamiana* plants under stress when inoculated with the same fungal strains that we used in the present study (Dastogeer et al., 2017a). This could be because of the differences in plant genotype and their inherent drought tolerance level suggesting that the endophyte-plant interaction is context dependent. Increased root biomass of endophytic plants under stress could be a mechanism to uptake water from a greater area and increase tolerance. A similar mechanism could be associated with virus-infected plants in the current study, which was supported by the findings that virus infection did not change plant root biomass under stress, although it reduced the shoot biomass substantially under stress.

As can be expected, the RWC of *Nicotiana* seedlings significantly diminished under stress, while colonization with either of the fungal strains enhanced the leaf water levels to a small extent. This is in accordance with previous studies. For example, inoculation with a strain of *Chaetomium globosum* resulted in an increased water content of wheat leaves subjected to water stress as compared to non-endophytic counterparts (Cong et al., 2015). Also, *Trichoderma atroviride* colonization in maize (Guler et al., 2016) and *T. hamatum* colonization in cacao (Bae et al. 2009) showed a relative increase in water status under stress conditions. In similar research, it was shown that endophytic plants use significantly less water than non-endophytic plants (Chepsergon et al. 2014). Martinez-Medina et al. (2014) also showed that endophytic *T. hamatum* drew water from deeper soil to enhance plant water potential. Endophytes could also help plants reduce the losses of water as the stress progresses to better the plant performance by reducing transpiration and through osmotic and metabolite adjustment (Dastogeer et al., 2017b; Morsy et al., 2010; Rodriguez et al., 2010; Waller et al., 2005).

Reduction in chlorophyll content under water stress regimes is the main cause of inactivation of photosynthesis (Shukla et al., 2012). Root colonization with the fungal endophyte E-162 alleviated the water stress effect by improving chlorophyll pigment contents in *N. benthamiana* seedlings. A similar result has been reported by Bae et al. (2009) who reported an increase in chlorophyll content in seedlings colonized by *T. hamatum*. Harman et al. (2004) reported that a strain of *T. harzianum*

increased leaf greenness in maize, which enhanced the vigour when an adequate carbon source was available for plant development. In our study, the isolate E-284 did not show any effect on chlorophyll content, while a positive effect of fungal endophytes on plant chlorophyll is common (Bayat et al., 2009; Guler et al., 2016; Khan et al., 2013; Khan et al., 2015; Mastouri et al., 2012; Shukla et al., 2012; Sun et al., 2010b; Waqas et al., 2012; Zhang and Nan, 2007), neutral or negative (Pandey et al., 2016; Ren et al., 2011; Tian et al., 2015b). It is understandable that virus infection severely reduces plant chlorophyll content (Dai et al., 2009; Guo et al., 2005; Plattt et al., 1979). However, it is interesting that in our study, water stress did not influence chlorophyll content in virus infected plants. One possible explanation is that initial plant drought response includes wilting of lower leaves and plant diverts most available water and energy to the upper leaves from where we measure chlorophyll to increase photosynthesis to support growth. The virus accumulation may not keep pace with the activity of these leaves as they can do in the well-watered plant leaves resulting in slower symptom expression (such as yellowing) and hence the disruption of chlorophyll. However, we did not come across any literature explaining this phenomenon and this may be an interesting area of future studies.

A higher level of EC in the plant under water stress is an indication of damage to the cell membrane. Higher leakage of solutes could be associated with increased H<sub>2</sub>O<sub>2</sub> production and lipid peroxidation under stress causing membrane destruction and metabolic toxicity leading to higher solutes leakage (Deshmukh et al., 1991; Dionisio-Sese and Tobita, 1998; Premchandra et al.). An increased EC in non-endophytic plants suggests more tissue damage, while this is reduced by the presence of endophytic fungi under stress conditions. Endophyte-mediated reduced EC in plants under stress has been reported in other studies (Bayat et al., 2009; Khan et al., 2013; Shukla et al., 2012; Tian et al., 2015b).

#### **4.2 Osmolytes under water stress**

Increased sugar accumulation in plants in response to water stress has been documented (Assuero et al., 2006; Ren et al., 2006). Accumulation of soluble sugars in plants is an adaptive response to stress, which functions as an osmotic adjustment balance (Ren et al., 2006). Research on the influence of fungal endophytes on plant sugar accumulation under water deficit stress reported variable results. For example, endophytes may increase (Assuero et al., 2006; Ren et al., 2006; Richardson et al., 1992; Yang et al., 2014) or decrease (Hill et al., 1990) or exert no apparent influence (Cong et al., 2015; Hill et al., 1990) sugar levels in stressed plants, depending on the host species, breeds and genotypes. Higher sugar accumulation could contribute to the osmotic potential of endophytic plants under stress, which might be initiated by altered carbohydrate balance or by carbohydrate re-metabolization from sugars of senescing leaves (Assuero et al., 2006).



Stressed increased plant soluble proteins. This could be related to their roles in permeation and antioxidation, for example, antioxidant enzymes, phytohormone receptors, etc (Yang et al., 2014). Endophyte-mediated increased soluble proteins and various amino acids in response to water stress has also been reported (Bae et al., 2009; Yang et al., 2014).

Stress induced proline accumulation is common in plants, although its role as an osmoprotectant is still debated (Delauney and Verma, 1993; Serraj and Sinclair, 2002b). Proline accumulation may or may not have any relation with plant drought tolerance (Cha-Um et al., 2010; Hien et al., 2003; Roy et al., 2009). Similar to our findings, endophyte-induced increase in proline was described from drought stressed grass species (Abernethy and McManus, 1998; Bandurska and Józwiak, 2010; Elbersen and West, 1996; Malinowski and Belesky, 2000a; Nagabhyru et al., 2013). The higher proline accumulation in the endophytic plant could be due to the role of endophytes under stress conditions to shield plants from the severity of damage through increased accumulation of osmoprotectants (such as proline). It was noted that virus infection increased proline in both watered and stressed plants. This might be because virus infection put the plant under stress, stimulating proline accumulation which was intensified even more under water stress.

#### **4.3 ROS and antioxidant enzymes under water stress**

When photosynthetic tissue is exposed to paraquat, it generates superoxide ions and causes photobleaching through the reduction of the electron transfer from the plants photosystem I and oxidation by molecular oxygen (Vaughn and Duke, 1983). ROS production is associated with early events in the plant stress response mechanism. The current study indicated that water stress tolerance in endophytic or virus inoculated plants correlated with reduced ROS activity. Higher ROS synthesis is common to all stresses because of stress-induced metabolic imbalances (Apel and Hirt, 2004; Vaughn and Duke, 1983). Decreased ROS activity in endophytic or YTMV-infected plants could be achieved by improved ROS scavenging of antioxidation systems, which correlate strongly with stress tolerance and may play a critical role in the process.

Plants tend to produce additional ROS in response to various environmental stimuli. Various pathways are involved in detoxifying ROS in plants (Mittler, 2002). Plant cellular antioxidative enzymes are produced to scavenge more  $H_2O_2$  (Tran et al., 2012). While the interaction between endophytes and the elements of defence mechanisms is not well-understood, activities of CAT, POD, and PPO were more significantly increased in endophytic plants than in non-endophytic plants under water stress. These enzymes counteract ROS induced oxidative damage. Peroxidase and polyphenol oxidase protect cells against the toxic effects of  $H_2O_2$  by catalysing its decomposition through the oxidation of phenolic osmolytes (Elmi and West, 1995). Our results correlate with those of others that suggest these enzyme activities are increased in endophytic plants challenged with abiotic and biotic stress (Harman, 2011; Khan et al., 2013; Waller et al., 2005). Endophyte-inoculated plants recruited

higher amount of oxidative stress enzymes that successively protect plants against ROS formation and membrane damage under stress (Baltruschat et al., 2008; Guler et al., 2016; Harman, 2006; Hashem et al., 2014; Khan et al., 2014; Khan et al., 2013; Mastouri et al., 2012; Shukla et al., 2012; Waller et al., 2005). Virus-induced changes CAT, POD and PPO activity in plants have been reported (Amoako et al., 2015; Buonauro and Montalbini, 1993; Chatterjee and Ghosh, 2008; Madhusudhan et al., 2009; Riedle-Bauer, 1998; Srivastava and Singh, 2010). In the current study, the activity of all these antioxidant enzymes was increased in the presence of the virus. This could be one of the mechanism the plant uses for virus resistance. The plant we used is a wild accession of native Australian *N. benthamiana* which responded with moderate symptoms to YTMV infection. The virus produces mosaic or mottling symptoms on leaves of *N. benthamiana* plants, but is not lethal to them. Under water stress, the virus resulted in a more pronounced increase in these enzymes. We hypothesise that when the plant was challenged with water stress and YTMV simultaneously, the plant produces more antioxidant enzymes to protect it from increased ROS production. We assumed that the effect of fungal infection was masked by virus infection and therefore its influence could not be distinguished from the effect of the virus.

#### 4.4 Drought related gene expressions

It is known that catalase is one of the key enzymes participating in the regulation of H<sub>2</sub>O<sub>2</sub> in cells. The upregulation of NbCAT3 is correlated with increased CAT activity during water stress as well as virus infection. This could be a plant strategy to shield the toxic influence of elevated ROS under stress. At the same time, activation of CAT may be beneficial for the virus, by reducing the efficiency of the plant defence system. It is still unclear if activation of NbCAT3 is a consequence of viral infection, or if it is a common response to ROS upregulation under any stress stimulus. Some plant viruses are known to influence the expression of antioxidant defence-related genes (Kogovšek et al., 2010; Pompe-Novak et al., 2006; Yergaliyev et al., 2016).

Several transcription factors, MYB, MYC, DREB, and ZFP, have roles in plant development and stress tolerance (Kizis et al., 2001; Mengiste et al., 2003; Meshi and Iwabuchi, 1995; Narusaka et al., 2003; Xiang et al., 1997). The MYB family of proteins is large, functionally diverse and represented across all eukaryotes. Different MYB transcription factors have been characterised in many plant species, and their involvement in drought responses have been described (Ambawat et al., 2013; Baldoni et al., 2015). NbPHAN, a MYB transcriptional factor, was found to regulate leaf development and affects drought tolerance in *N. benthamiana* plants (Huang et al., 2013).

The WRKY transcription factors are unique to plants (Eulgem et al., 2000; Kalde et al., 2003). The WRKY proteins have been implicated in cellular defence against a variety of biotic and abiotic stressors, including drought and salt (Jiang and Deyholos, 2006; Tripathi et al., 2014). In the present

study, we found that NbWRKY, an NTEIG-D homolog (WERKY-like gene), is upregulated under stress and was upregulated under fungus infection. The earlier study suggests that NbWRKY overexpressed in *N. benthamiana* plants under water stress (Archana et al., 2009).

Most genes investigated in this study were upregulated in response to water stress in fungus- and virus-colonized seedlings. Endophyte-induced upregulation of drought related genes was reported in *Piriformospora indica*-infected *Arabidopsis* seedlings (Sherameti et al., 2008a; Sherameti et al., 2008b; Sun et al., 2010a; Xu et al., 2017). It is likely that a more comprehensive analysis will uncover more fungus- and virus-responsive genes and proteins involved in water stress tolerance.

Taking these results together, both fungal endophytes and the virus may confer water stress tolerance to *N. benthamiana* seedlings by increased accumulation of sugar, protein and proline as osmolytes, increased antioxidative enzyme activity, reduced membrane damage, and enhanced expression of drought-related genes. Plant drought tolerance is a complex trait. Roles of compatible solutes in plant drought stress tolerance have been discussed elaborately. At physiological level, osmotic adjustment is an adaptive mechanism involved in drought tolerance and permits the maintenance of turgor pressure under stress conditions. Also, these osmoprotectants (sugars, prolines, proteins etc) and antioxidant enzymes detoxify adverse effect of reactive oxygen species and alleviate drought stresses in plants (reviewed by Singh et al. 2015). Our results suggest, the fungal endophytes and the virus helps plant increase plant biomass, chlorophyll, RWC and osmoprotectants as well as antioxidant enzymes and thus enhance the adaptive drought tolerance mechanisms in plant.

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## Figures

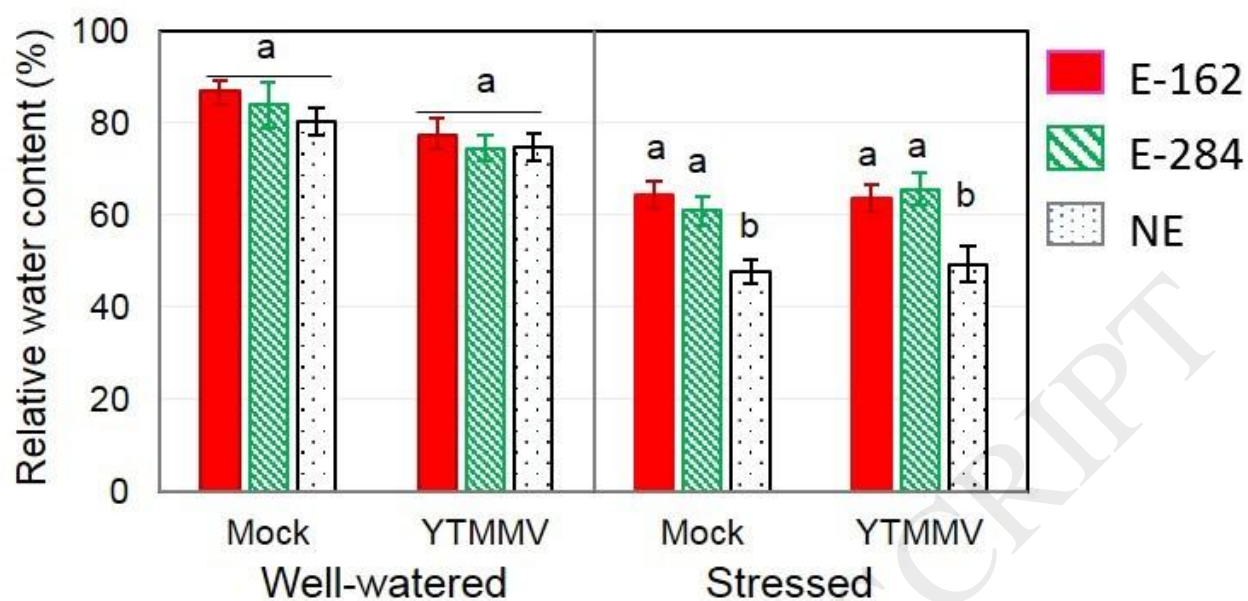


Fig 1. Relative water content of *N. benthamiana* leaf at 8 d after stress application. Bar indicates mean with standard error (SE), N=12. Bar with different letters a significantly different at  $p \leq 0.05$  under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's honestly significant difference (HSD) test. The E-162 indicates a plant inoculated with the fungal strain E-162, E-284 indicates a plant inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

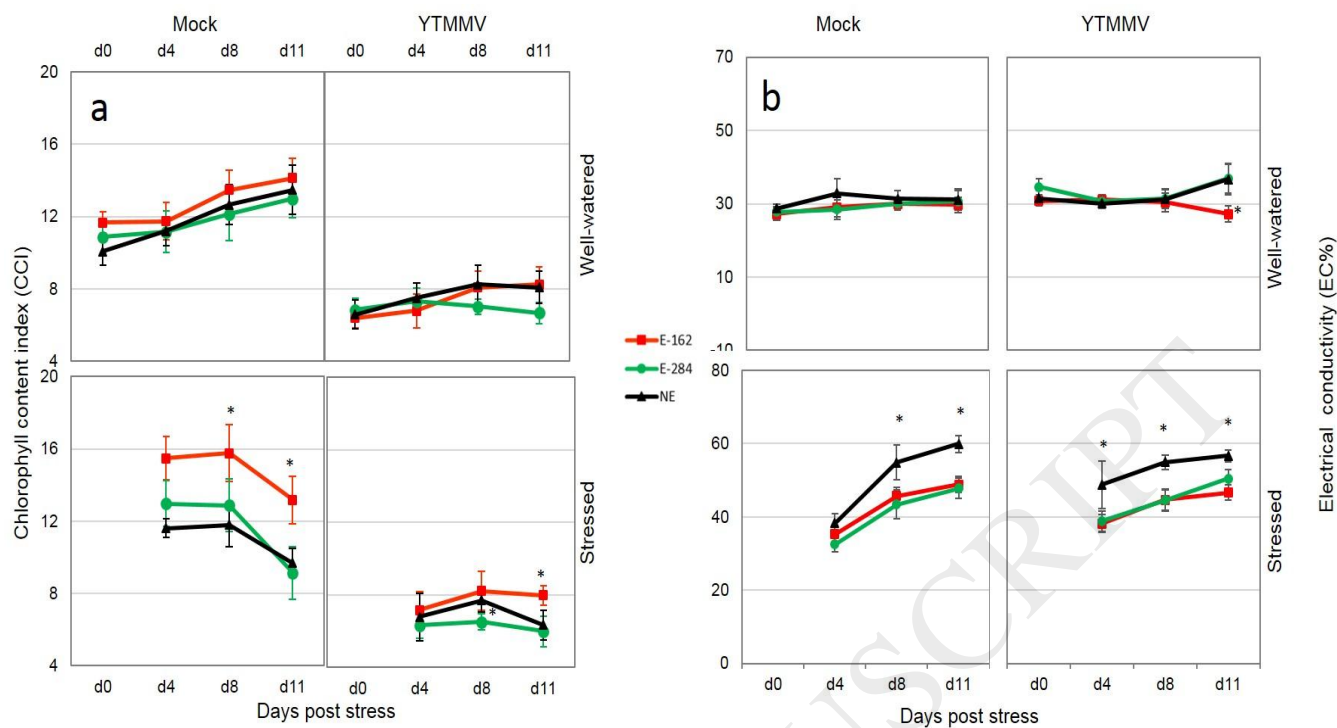


Fig. 2. (a) Chlorophyll Content Index and (b) Electrical conductivity values of *N. benthamiana* leaf at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=6. The “\*” symbol above any line indicates the value is significantly different ( $p \leq 0.05$ ) from the other treatments at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey’s HSD test. The E-162 indicates plants inoculated with the fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

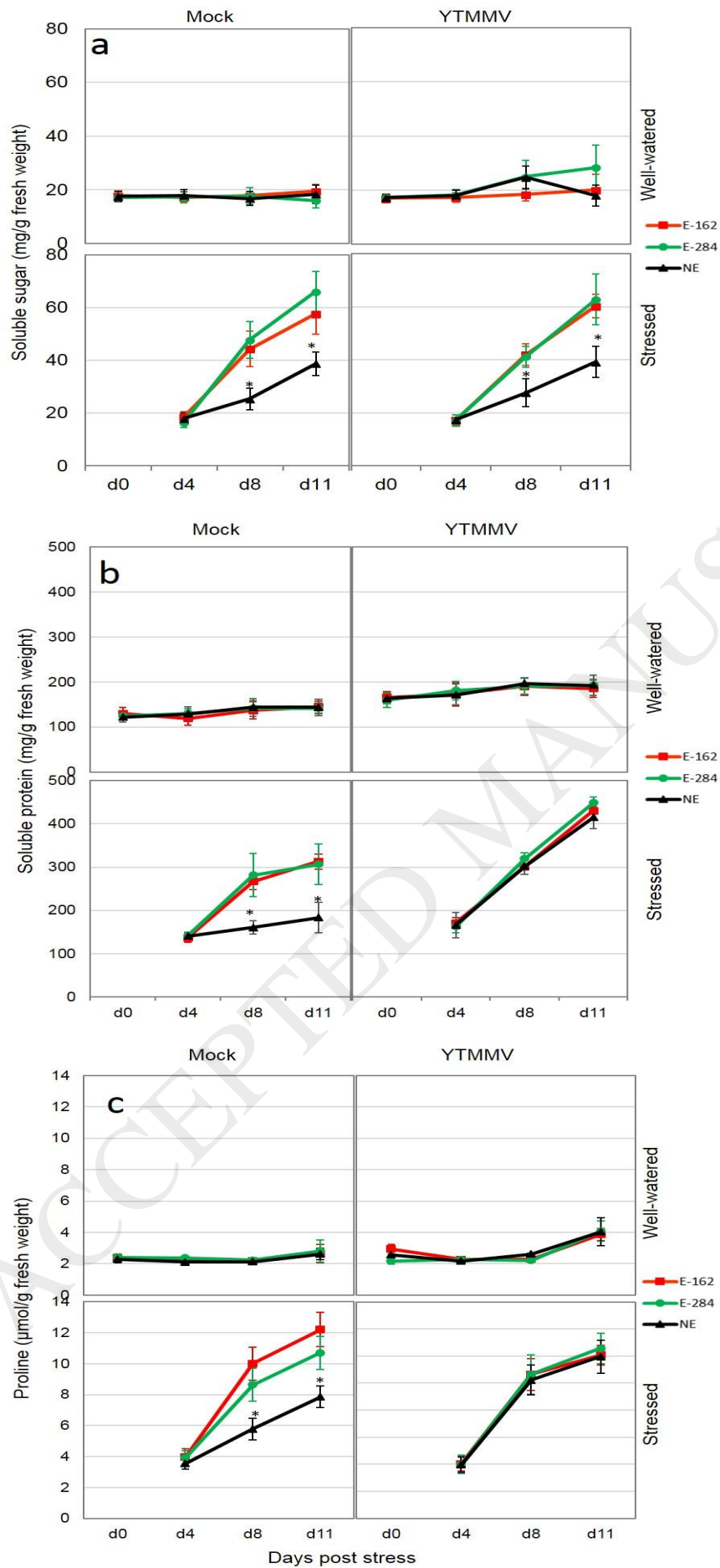


Fig. 3. (a) Soluble sugar (b) Soluble protein and (c) Proline content of *N. benthamiana* leaves at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=12. The “\*” symbol above any line indicates the value is significantly different ( $p \leq 0.05$ ) from the other treatments at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey’s HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

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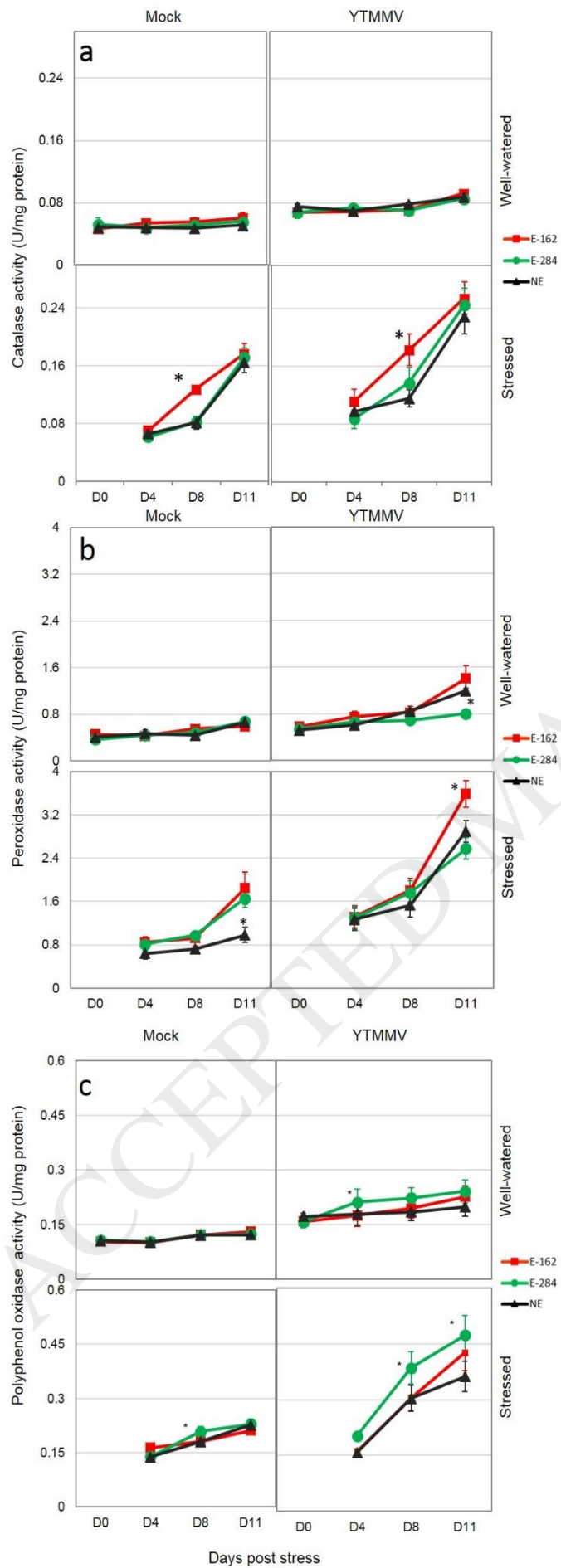




Fig. 4. Activity of (a) catalase, (b) and peroxidase (c) polyphenol oxidase enzymes in *N. benthamiana* leaf at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=12. The “\*” symbol above any line indicates the value is significantly different ( $p \leq 0.05$ ) from the other treatment at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey’s HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus and YTMMV = plants inoculated with YTMMV (yellowtail flower mild mottle virus).

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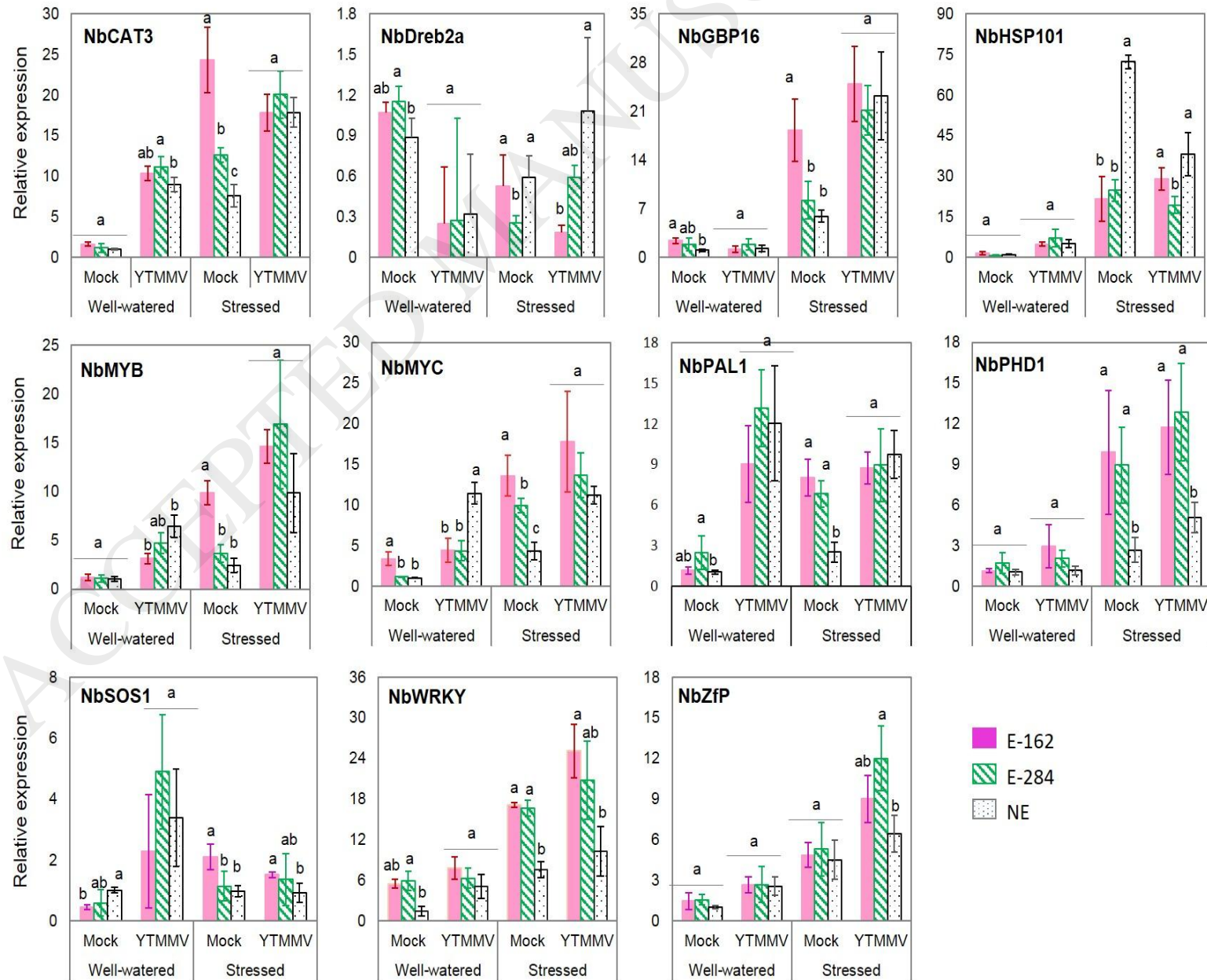


Fig 5. Relative changes in expression of drought related genes in *N. benthamiana* leaves under water stress after 8 d. The bars indicate standard error (SE), N=3. Bars with different letters are significantly different ( $p \leq 0.05$ ) under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's HSD test. E-162 indicates plant inoculated with fungal strain E-162, E-284 indicates plant inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plants inoculated with YTMMV (yellowtail flower mild mottle virus).

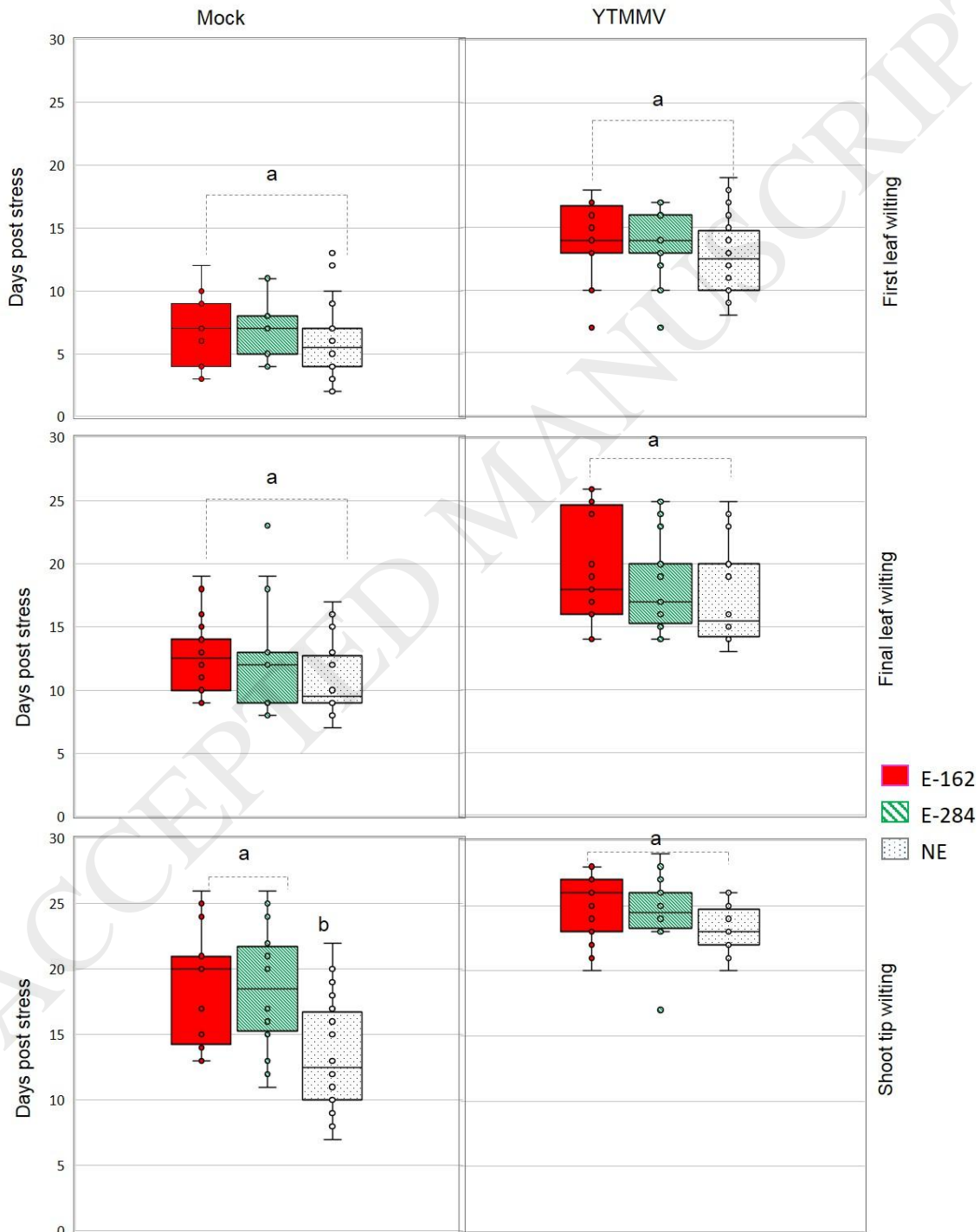


Fig. 6. Wilting behaviour of *N. benthamiana* under water stress. The bars indicate standard error (SE), N=12. Bars with different letters are significantly different ( $p \leq 0.05$ ) as obtained by Tukey's HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

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Table 1: Primers used for quantitative reverse transcriptase-polymerase chain reaction analysis of *Nicotiana benthamiana* drought associated genes

Gene Code	Description	GenBank Accession	Forward Primer (5'-3')	Reverse Primer (3'-5')
<b>NbPAL1</b>	<i>N. benthamiana</i> cDNA similar to Phenylalanine ammonialyase 1	JK739025.1	CCCATGTTTGCCTCTCCGTG	GCCCGTTGAAACACCTTGC
<b>NbMYB</b>	<i>N. benthamiana</i> cDNA similar to Myb-related transcription factor	JK739024.1	TGGCCTGGAGACTGATAACG	GATGTAGGTGGTGGTGGTGG
<b>NbMYC</b>	<i>N. benthamiana</i> cDNA similar to MYC related transcription factor	JK739021.1	GATCAATGCCAAACTGGAAGC	GACTTAGCGGTGGTTAAGAGTC
<b>NbGBP16</b>	<i>N. benthamiana</i> cDNA similar to DNA-binding protein GBP16	JK739018.1	TTGAGGCTTGTGAGACCAGG	GGCTTTCCTTCACCAGTGCT
<b>NbSOS1</b>	<i>N. benthamiana</i> cDNA similar to SOS1 mRNA for Na <sup>+</sup> /H <sup>+</sup> antiporter	JK739016.1	GACAAGGGCAAGGGTGATTA	CGTCTTCGCTATTATCCCCTC
<b>NbPDH1</b>	<i>N. benthamiana</i> cDNA similar to Proline oxidase/dehydrogenase 1 (PROX1)	JK739015.1	GCCGTTTAAACTCAGCTCCG	GAACGCCGTAGAACAGCTCC
<b>NbCAT3</b>	<i>N. benthamiana</i> cDNA similar to Catalase (CAT3)	JK739008.1	GGAGCAAATCATAGTCACGCC	GCAAGATGTCCTCAGGCCAG
<b>NbHSP101</b>	<i>N. benthamiana</i> cDNA similar to Heat shock protein 101 kDa (HSP101)	JK739013.1	CTGAGGCAGCTCTAGATTTTCATAC	ATTAGGAGATGGTTGGAGAGGA
<b>NbZfp</b>	<i>N. benthamiana</i> zinc finger protein mRNA	AY899938.1	AGGCACAAAACGGAAAACAC	CTGGCATTGATTGAGCAGA
<b>NbDreb2a</b>	<i>N. benthamiana</i> mRNA for transcription factor DREB2a	FN649467.1	TGAAAGCGCTTGAACCTTTT	CTACACGGCCCATAGGGTTA
<b>NbWrky</b>	<i>N. benthamiana</i> cDNA 5- similar to WRKY transcription factor 44 (WRKY DNA-binding protein 44)	EH367381.1	GTCTGAGGCATCCAAGACAA	CCTCATTCCGGCACAATAAATG
<b>NbActin</b>	<i>N. benthamiana</i> actin (act) mRNA, act-b allele	JQ256516.1	GAGCGGGAAATTGTCAGGGA	GAAACGCTCAGCACCAATGG

Table 2: Results of the three-way ANOVA of relative water content and biomass data of *N. benthamiana* plants subjected to water stress and inoculated with fungal endophytes and virus<sup>1</sup>.

	<b>RWC (n=12, error df=132)</b>	<b>Biomass traits (n=6, error df=60)</b>		
	F value	Shoot	Root	Root: Shoot
		F value	F value	F value
<b>WT</b>	121.51***	35.68***	9.19**	27.09***
<b>VI</b>	2.66	32.36***	25.77***	0.00
<b>EI</b>	10.42***	0.15	0.59	1.28
<b>WT×VI</b>	7.01**	4.92*	8.45**	6.06*
<b>WT×EI</b>	4.46*	0.22	0.93	3.55*
<b>VI×EI</b>	0.25	0.02	0.07	0.64
<b>WT×VI×EI</b>	0.32	0.03	0.28	0.53

<sup>1</sup>WT= Water treatment, EI= Endophyte inoculation, VI= Virus inoculation, RWC= Relative water content. The “\*”, “\*\*” and “\*\*\*” symbols indicate the F-value is significant at  $p \leq 0.05$ ,  $p \leq 0.01$ ,  $p \leq 0.001$ , respectively.

Table 3: Effect of fungal endophyte and virus infection on biomass and ROS sensitivity in water-stressed *N. benthamiana* plants at d8\*

Water treatment	Biomass trait	Shoot (mg)		Root (mg)		Root: Shoot		ROS	
	EI	Mock	YTMMV	Mock	YTMMV	Mock	YTMMV	Mock	YTMMV
WS	E-162	273.82±53.9aA	160.03±31.6aA	51.42±6.1aA	38.17±8.1aA	5.20±0.7bA	4.18±0.51aA	7.6±0.87aA	5±0.84aB
	E-284	241.87±34.2aA	131.23±25.8aB	49.78±7.8aA	31.23±5.8aA	4.94±0.2bA	4.65±0.67aA	8.4±0.51aA	3.8±0.58aB
	NE	243.03±23.3aA	136.53±22.2aB	31.58±1.7bA	27.60±6.1aA	7.80±0.9aA	5.66±0.89aA	10.4±0.51bA	4.8±0.58aB
WW	E-162	502.20±58.9aA	269.50±32.7aB	74.72±15.1aA	29.48±3.9aB	7.32±0.8aA	9.33±0.8aA	1.4±0.51aA	0.6±0.24aA
	E-284	536.83±135.7aA	273.60±59.5aA	77.75±21.0aA	37.33±8.3aA	7.29±0.5aA	7.94±0.9aA	2.2±0.86aA	1±0.32aA
	NE	501.03±53.2aA	242.77±17.4aB	78.27±9.5aA	32.23±2.9aB	7.12±1.4aA	7.79±.07aA	0.8±0.37aA	0.8±0.37aA

\*The values with different letters are significantly different ( $p \leq 0.05$ ) as obtained by Tukey's honestly significant different test; small letters compare across column s separately under well-watered (WW) and water stress (WS) condition and the capital letters compare across rows between Mock and YTMMV. E-162=plants inoculated with fungal strain E-162, E-284=plants inoculated with fungal strain E-284, NE=No fungal inoculation, Mock = in absence of virus and YTMMV = plants inoculated with YTMMV; ROS=reactive oxygen species.

Table 4: F-values obtained from the three-way ANOVA of data of various parameters under well-watered and stressed conditions.<sup>1</sup>

		df	EC	Proline	Protein	Sugar	Catalase	Peroxidase	Polyphenol oxidase
Well-watered (n=12, error df=336)	VI	1	9.07*	9.65*	46.19***	4.18*	145.55***	49.82***	111.83***
	EI	2	1.3	0.29	0.05	0.59	0.02	1.34	0.62
	Day	3	1.77	12.69***	3.35*	1.66	7.60***	16.94***	6.33***
	VI×EI	2	0.89	0.04	0.001	1.55	1.51	0.55	0.51
	VI×Day	3	0.61	4.29*	0.3	1.51	1.78	2.4	1.59
	EI×Day	6	0.96	0.36	0.11	0.37	0.66	0.74	0.46
	VI × EI × Day	6	0.63	0.46	0.05	0.76	0.46	1.41	0.4

<b>Stressed (n=12, error df=198)</b>	VI	1	1.02	11.87**	60.52***	0.13	50.14***	124.45***	94.03***
	EI	2	14.31***	3.32*	7.32**	13.42***	5.91*	6.54**	4.50*
	Day	2	37.29***	101.75***	103.01***	76.25***	107.59***	75.30***	55.91***
	VI×EI	2	0.86	5.01*	4.08*	0.21	0.33	1.86	2.68
	VI×Day	2	2.57	2.33	12.85***	0.08	2.5	11.71***	16.15***
	EI×Day	4	0.36	0.56	1.98	3.86**	1.69	2.43*	0.48
	VI × EI × Day	4	0.79	0.99	1.18	0.17	0.099	1.27	0.54

<sup>1</sup>EI= Endophyte inoculation, VI= Virus inoculation, EC= Electrical conductivity. The “\*”, \*\* and \*\*\*” symbols indicate the F-value is significant at  $p \leq 0.05$ ,  $p \leq 0.01$ ,  $p \leq 0.001$ , respectively.



Table 5. F values obtained from three-way ANOVA of the data of relative expression of the *N. benthamiana* drought associated genes. \*

Gene Code	WT	VI	EI	WT×VI	WT×EI	VI×EI	WT×VI×EI
Degrees of freedom	1	1	2	1	2	2	2
NbCAT3	111.25	36.75	6.78	6.16	4.34	6.24	6.34
NbDreb2a	0.9	5.45	1.00	13.29	1.95	1.55	0.79
NbGBP16	75.87	11.55	1.92	12.74	1.63	1.01	0.57
NbHSP101	173.96	1.73	19.57	10.82	21.33	6.98	6.9
NbMYB	22.9	19.25	0.96	3.04	2.63	1.15	0.75
NbMYC	32.4	14.05	1.85	0	5.42	2.07	0.54
NbPAL1	0.68	30.86	0.58	7.51	0.96	1.39	0.36
NbPDH1	28.45	1.82	4.16	0.59	2.53	0.04	0.17
NbSOS1	1.93	5.98	0.25	7.25	1.21	0.76	0.25
NbWrky	55.94	5.81	10.86	0.92	3.44	0.34	0.45
NbZfP	43.61	13.74	1.8	3.64	1.21	0.68	1

\*The Ct value for each gene was normalised with the Ct value of NbActin and relative expression value was obtained using  $2^{\Delta\Delta Ct}$  method. A three-way ANOVA was performed to detect whether the effect of WT= water treatment, VI=virus inoculation and EI= Endophyte inoculation and all possible interactions between them were significant. The values provided are F-ratio. The cells marked with dark are significant at  $p \leq 0.001$  and those with grey and light grey are significant at  $p \leq 0.01$  and  $p \leq 0.05$ .