Chapter 3
Pre-Screening of Banana Genotypes for Fusarium Wilt Resistance by Using an *In Vitro* Bioassay

Yuanli Wu and Ganjun Yi

Abstract In the process of breeding and selection of banana for resistance to Fusarium wilt, it is important to conduct an efficient resistance screening test by artificial inoculation with *Fusarium oxysporum* f. sp. *cubense* (Foc) Tropical Race 4. So far, there are two types of early bioassays for screening *Musa* genotypes against Foc: a greenhouse and an *in vitro* bioassay. The most commonly used greenhouse bioassay is a pot-based system followed by a hydroponic system. Here we describe an *in vitro* bioassay characterized by *in vitro* inoculation of rooted banana plantlets grown on medium consisting of half-strength MS macronutrients and MS micronutrients. The disease response and evaluation results obtained through this *in vitro* bioassay correlates with that from a greenhouse screen and/or field evaluation. Given the importance of *in vitro* cell and tissue culture techniques for banana (mutation) breeding, promising resistant clones could be screened directly. This *in vitro* bioassay is a totally contained system compared with greenhouse methods and does not require an acclimatization step, thereby improving banana breeding efficiency. The *in vitro* pre-screening protocol and bioassay for Fusarium wilt resistance presented here is fast, space-effective, and accurate.

Keywords Bioassay · *Fusarium oxysporum* f. sp. *cubense* · *Musa* · Resistance

1 Introduction

Fusarium wilt or Panama disease caused by the pathogenic fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is one of the most destructive diseases of banana and is found in all areas where banana is grown (Ploetz 2015). The soil-borne

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pathogen infects the roots of banana plants and colonizes the xylem vessels, which leads to typical external symptoms such as wilting and yellowing of the foliage, eventually leading to plant mortality. Currently, there is still a lack of economically viable measures for managing Fusarium wilt in an infected field (Dita et al. 2018). It is generally accepted that breeding and selection for disease resistance is the only effective and sustainable management option.

Promising resistant clones acquired through conventional and non-conventional breeding techniques should be screened for resistance to Fusarium wilt using artificial inoculation with Foc. So far, there are two types of early bioassays for screening *Musa* genotypes against Foc: a greenhouse and an *in vitro* bioassay (Wu et al. 2010; Ghag et al. 2012; Hu et al. 2013). The most commonly used greenhouse bioassay is a pot system (Dita et al. 2011; Ribeiro et al. 2011; Li et al. 2015; Reboucas et al. 2018; Zhang et al. 2018; Zuo et al. 2018) followed by a hydroponic system.

Although some progress has been achieved, attempts at developing new banana genotypes resistant to Fusarium wilt using conventional breeding techniques face significant hurdles, mainly because most cultivars of *Musa* AAA Cavendish subgroup are sterile and seedless (Ortiz 2013). Nowadays, non-conventional breeding approaches for banana improvement such as somaclonal variation and genetic transformation have received more attention. Somaclonal variation caused by long-term *in vitro* propagation is considered an important source of genetic variability, through which several tolerant clones have been acquired (Hwang and Ko 2004). In addition, a genetic transformation protocol has been well established in different banana genotypes, which can be used to create transgenic plants resistant to Foc Tropical Race 4 (Foc TR4) (Dale et al. 2017). Given the fact that non-conventional breeding techniques are based on banana cell and tissue culture, promising resistant clones could be screened directly using an *in vitro* bioassay. Based on the modification of previous work (Wu et al. 2010), we present here a pre-screening protocol for Fusarium wilt resistance by using an *in vitro* bioassay that is fast, space-effective, and accurate.

The *in vitro* bioassay is characterized by *in vitro* inoculation of rooted banana plantlets grown in a half-strength MS medium without a carbon source. Twenty-four days after inoculation with Foc TR4 at 10^6 conidia/ml, the disease severity was rated on a scale from 1 to 6 (Wu et al. 2010). Results of the disease score were then subjected to ordinal logit model analysis, which is also known as proportional odds model (McCullagh 1980). According to symptom rating probability distribution, the reaction of banana genotypes against Fusarium wilt was divided into five categories: highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), and highly susceptible (HS). Compared with the greenhouse bioassay, this *in vitro* bioassay is a totally closed system. Since acclimatization of *in vitro* plantlets is not required, the application of the bioassay improves banana breeding efficiency.

2 Materials

2.1 Medium for Interaction System (MIS)

1. MS (Murashige and Skoog 1962) macronutrients and micronutrients (*see* Note 1).
2. Tissue culture grade water.
3. Gelling agent (*e.g.* agar).
4. Analytical balance.
5. Weighing trays.
6. Spatula.
7. Beakers (500 ml and 1000 ml).
8. Magnetic stir bar.
9. Hot plate.
10. pH meter.
11. NaOH (1 N).
12. HCl (1 N).
13. Erlenmeyer flasks (150 ml).
14. Ventilated sealing film for Erlenmeyer flask (12×12 cm).
15. Rubber bands.

2.2 Plant Material Preparation

1. High-quality *in vitro* plantlets which have been cultured on rooting medium for 2 weeks (Wu et al. 2005).
2. Sterile MIS medium.
3. Forceps.
4. Scalpel handle.
5. Scalpel blades.

2.3 Inoculum Preparation

1. Five-days-old PDA (potato dextrose agar) plate culture of Foc TR4 strain GD-13 (VCG 01213/16, ACCC 37997), which was isolated from a diseased Cavendish banana plant in Guangdong Province, PR China (*see* Note 2).
2. Electron Microscopy Sciences (EMS) Rapid-Core (6.0 mm).
3. Potato Dextrose Water (ready-to-use).
4. Erlenmeyer flask (500 ml).

5. Orbital shaker.
6. Cheesecloth.
7. Petri dishes.
8. Short-stem funnel (60 mm).
9. Bottles (100 ml).
10. Beakers (500 ml).
11. Forceps.
12. Haemocytometer.

2.4 *In Vitro* Inoculation

1. Filter paper.
2. Petri dish.
3. Sterile distilled water.
4. Sterile centrifuge tube (50 ml).
5. Beakers (100 ml).
6. Forceps.
7. Disposable pipettes.

3 Methods (*See* Note 3)

3.1 *Preparation of MIS Medium*

1. Prepare stock solutions of MS macronutrients (20×) and micronutrients (200×).
2. For 1 liter of MIS medium, weigh 6 g of agar in 400 ml of tissue culture grade water.
3. Heat while stirring until the solution is homogenous and clear.
4. In a separate beaker containing 400 ml water mix 25 ml stock solution of MS macronutrients (20x) and 5 ml stock solution of MS micronutrients (200x), stir the mixture.
5. Mix the solution prepared in step 3 with the solution prepared in step 4.
6. Add water to a final volume of 1 liter, while stirring adjust the pH to 5.8 using NaOH and HCl.
7. Dispense 50 ml of the medium into Erlenmeyer flasks.
8. Close each Erlenmeyer flask over the top with a ventilated sealing film and a rubber band.
9. Sterilize the medium for 20 min at 120 °C.
10. Allow medium to cool prior to use.
11. Store for up to a week in a cold room.

3.2 Plant Material Preparation

1. Autoclave forceps and scalpel.
2. In a laminar flow bench, remove the blackened part at the base of the rooted plantlets (the height of the pseudostem is 3.5–4.0 cm), shorten the roots to approximately 0.5 cm in length.
3. Transfer plantlets into Erlenmeyer flasks containing MIS, one plantlet per flask.
4. Maintain cultures at 25 ± 2 °C under a 12 h photoperiod with 50 μmol m^{-2} s^{-1} from cool white fluorescent lamps.
5. After 1–2 weeks of growth, select plantlets for *in vitro* inoculation: the height of the pseudostem should measure 4.5–5.0 cm, and the plantlet should possess more than two fully expanded leaves and at least three white roots.

3.3 Inoculum Preparation

1. Cut cheesecloth into round pieces (8 cm) and then place in a glass Petri dish.
2. Autoclave forceps, Erlenmeyer flask, Petri dish with cheesecloth pieces, short-stem funnel, bottle, and beaker.
3. In a laminar flow bench, dispense 200 ml of Potato Dextrose Water into Erlenmeyer flasks.
4. Punch out 5 mycelial plugs from the outer edge of the actively growing colony of Foc TR4 strain and transfer into an Erlenmeyer flask containing Potato Dextrose Water.
5. Place the Erlenmeyer flask on a rotary shaker (150 rpm) and incubate at 25 °C for 5 days.
6. In a laminar flow bench, insert funnel into bottle, then place four layers of cheesecloth into funnel.
7. Pour sporulation medium through four layers of cheesecloth to separate spores from mycelia.
8. After filtration, place cheesecloth pieces and funnel into beaker.
9. Determine conidia concentration in spore suspension using a haemocytometer.
10. Store spore suspension at 4 °C until *in vitro* inoculation.
11. Autoclave beaker with cheesecloth pieces and funnel.
12. Allow beaker to cool prior to disposing cheesecloth pieces.

3.4 In Vitro *Inoculation (*See *Fig. 3.1 and Note 4)*

1. Cut filter paper into small discs (5 mm) and then place in a glass Petri dish.
2. Autoclave forceps, Petri dish with filter paper discs, disposable pipettes, and beaker.

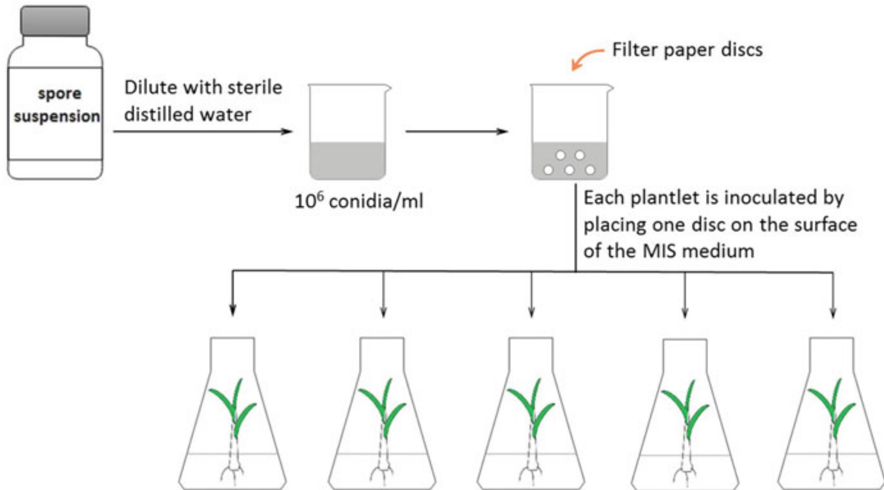

Fig. 3.1 *In vitro* inoculation protocol

3. In a laminar flow bench, adjust conidia concentration to 10^6 conidia/ml with sterile distilled water in a sterile centrifuge tube.
4. Pour the spore suspension (10^6 conidia/ml) into a beaker, then soak the filter paper discs in the spore suspension.
5. Inoculate each plantlet with Foc TR4 by placing one disc on the surface of the MIS medium.
6. Maintain cultures at 25 ± 2 °C under a 12 h photoperiod with 50 μmol m^{-2} s^{-1} from cool white fluorescent lamps for 24 days.
7. Autoclave beaker containing spore suspension for decontamination.

3.5 Disease Severity Rating and Statistical Analysis

1. Rate disease severity of the *in vitro* inoculated plantlets on a scale of 1 to 6: 1, no discoloration of the pseudostem; 2, $\leq$ 1/2 the height of the pseudostem discolored; 3, >1/2 the height of the pseudostem discolored and (or) leaf stalk discolored; 4, ≤50% of the leaves wilted or yellowed; 5, >50% of the leaves wilted or yellowed; and 6, the whole plantlet wilted (*see* Fig. 3.2).
2. After 24 days incubation assess disease severity by determining disease score.
3. Data collection: record the data for each banana plantlet and enter it into a spreadsheet (*e.g.* Microsoft Excel).
4. Model construction based on cumulative Logistic regression (*see* Note 5). Wherein the Logistic regression model accordingly contains five logit functions:

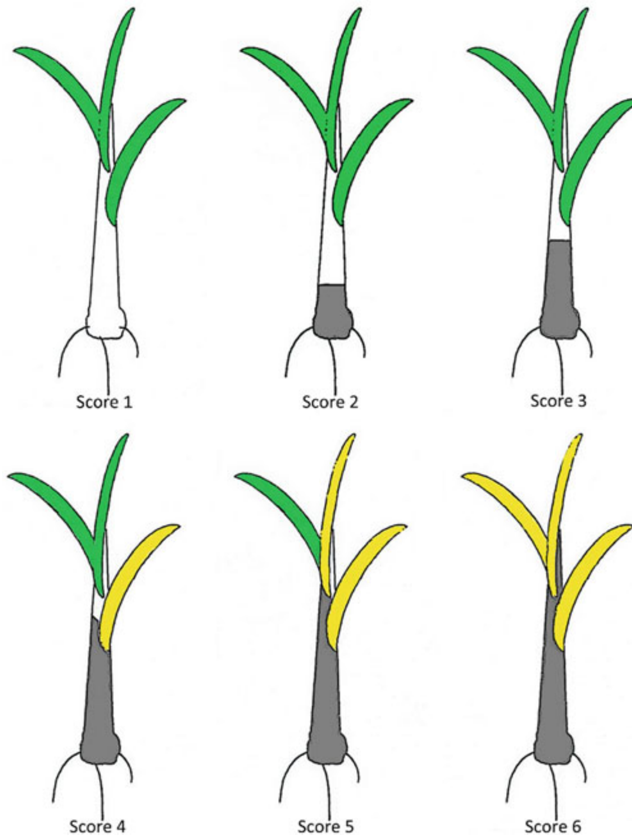

Fig. 3.2 A scale of 1–6 is used to measure disease severity of banana rooted plantlets 24 days after *in vitro* inoculation with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 at 10^6 conidia/ml

$$\ln\left(\frac{p_1}{1-p_1}\right) = \beta_{01} - \sum_{k=1}^{k} \beta_k x_k$$

$$\ln\left(\frac{p_1+p_2}{1-p_1-p_2}\right) = \beta_{02} - \sum_{k=1}^{k} \beta_k x_k$$

$$\ln\left(\frac{p_1+p_2+p_3}{1-p_1-p_2-p_3}\right) = \beta_{03} - \sum_{k=1}^{k} \beta_k x_k$$

$$\ln\left(\frac{p_1+p_2+p_3+p_4}{1-p_1-p_2-p_3-p_4}\right) = \beta_{04} - \sum_{k=1}^{k} \beta_k x_k$$

$$\ln\left(\frac{p_1+p_2+p_3+p_4+p_5}{p_6}\right)=\beta_{05}-\sum_{k=1}^{k}\beta_k x_k$$

Wherein p_1, p_2, p_3, p_4, p_5 and p_6 are event probabilities, which respectively represent disease grades of 1–6, and the basal level for comparison is grade 6; x_k ($k = 1,2\ldots, K$) represents banana cultivar; β_{0j} ($j = 1,2\ldots, 5$) represents an intercept term of regression; and β_k ($k = 1,2\ldots, K$) represents a regression coefficient; each logit function has the same coefficient term and different intercept terms, and the regression lines of each cumulative logit are parallel to each other.

The estimation method used for the Logistic regression model is a maximum likelihood method, according to the aforementioned Logistic model function designed for predicting the disease severity of the rooted plantlet of banana, the accumulated Logistic regression model obtained is described as follows:

$$y'=\alpha+\sum_{k=1}^{K}\beta_k x_k+\varepsilon$$

Wherein y' represents the disease incidence of the rooted plantlet of banana, α represents an intercept term; β_k ($k = 1,2\ldots, K$) represents a regression coefficient; x_k ($k = 1,2\ldots, K$) represents banana cultivar, and ε is an error term.

5. Calculation of cumulative probability: assigning respective values $y = 1, y = 2,..., y = 6$ to six disease grades, wherein a relationship among individual y values is $(y = 1) < (y = 2) < \ldots < (y = 6)$, and there are 5 demarcation lines for demarcating adjacent categories:

if $y' \le \mu_1$, $y = 1$
if $\mu_1 < y' \le \mu_2$, $y = 2$
if $\mu_2 < y' \le \mu_3$, $y = 3$
if $\mu_3 < y' \le \mu_4$, $y = 4$
if $\mu_4 < y' \le \mu_5$, $y = 5$
if $\mu_5 < y'$, $y = 6$

μ_j is a demarcation point for demarcating categories, and $\mu_1 < \mu_2 < \mu_3 < \mu_4 < \mu_5$.

The formula for calculating a cumulative probability value is as follows:

$$P(y\le j|x)=P\left(y'\le\mu_j|x\right)=\frac{1}{1+e^{-\left[\mu_j-\left(\alpha+\sum_{k=1}^{k}\beta_k x_k\right)\right]}}$$

Therefore, the probability value of the rooted plantlet of a banana cultivar in a certain disease grade can be obtained:

$$P\ (y = 1) = P\ (y \leq 1)$$
$$P\ (y = 2) = P\ (y \leq 2) - P\ (y \leq 1)$$
$$P\ (y = 3) = P\ (y \leq 3) - P\ (y \leq 2)$$
$$P\ (y = 4) = P\ (y \leq 4) - P\ (y \leq 3)$$
$$P\ (y = 5) = P\ (y \leq 5) - P\ (y \leq 4)$$
$$P\ (y = 6) = 1 - P\ (y \leq 5)$$

The sum of the probability values of each grade is 1, that is, $P\ (y = 1) + P\ (y = 2) + \ldots + P\ (y = 6) = 1$.

6. Open the spreadsheet at user interface of statistic software (*e.g.* IBM SPSS Statistics), then execute ordinal logit model analysis and obtain symptom rating probability distribution of each banana cultivar or clone. As an example, the result of statistical analysis is given in Fig. 3.3.

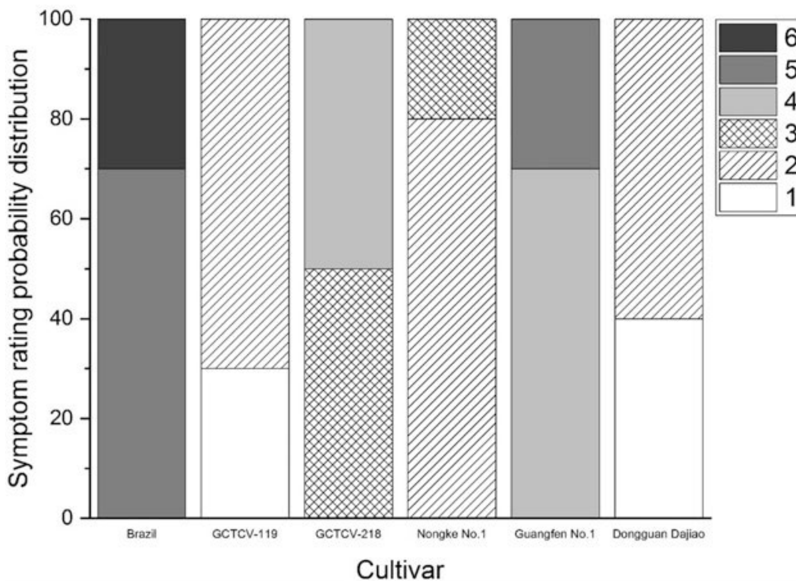

Fig. 3.3 *In vitro* inoculation of six banana cultivars with *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). Twenty-four days after *in vitro* inoculation of rooted plantlets ($n = 15$), disease symptoms were scored on an ordinal scale as illustrated: 1, no discoloration of the pseudostem; 2, ≤1/2 the height of the pseudostem discolored; 3, >1/2 the height of the pseudostem discolored and (or) leaf stalk discolored; 4, ≤50% of the leaves wilted or yellowed; 5, >50% of the leaves wilted or yellowed; and 6, the whole plantlet wilted. Control plantlets were treated in the same manner except that Foc TR4 was replaced with sterile distilled water. Data obtained from three independent experiments were subjected to ordinal logit model analysis, which is also known as proportional odds model

Table 3.1 Criteria for grading resistance against *Fusarium oxysporum* f. sp. *cubense*

Symptom rating probability distribution	Level of resistance to Fusarium wilt
P (y = 2) ≥50%, P (y = 1) ≤50%	HR
P (y = 2) ≥50%, P (y = 3) ≤50%	R
P (y = 3) ≥50%, P (y = 2) <50% or P (y = 4) ≤50%	MR
P (y = 4) ≥50%, P (y = 3) <50% or P (y = 5) ≤50%	S
P (y = 5) ≥50%, P (y = 4) <50% or P (y = 6) ≤50%	HS

Table 3.2 Screening of banana cultivars for resistance to Fusarium wilt under field, greenhouse, and *in vitro* conditions, respectively

	Level of resistance to Fusarium wilt		
Cultivar	Field Evaluation	Greenhouse Bioassay	*In vitro* Bioassay
Brazil (*Musa* AAA Cavendish subgroup)	HS (Huang et al. 2005)	S (Zuo et al. 2018)	HS
GCTCV-119 (*Musa* AAA Cavendish subgroup)	HR (Huang et al. 2005)	R (Zuo et al. 2018)	HR
GCTCV-218 (*Musa* AAA Cavendish subgroup)	MR (Huang et al. 2005)	–	MR
Nongke No.1 (*Musa* AAA Cavendish subgroup)	R (Liu et al. 2007)	–	R
Guangfen No.1 (*Musa* ABB Pisang Awak)	–	S (Zuo et al. 2018)	S
Dongguan Dajiao (*Musa* ABB group)	HR (Huang et al. 2005)	–	HR

7. Evaluate the disease resistance level of banana cultivars or clones according to their symptom rating probability distributions (*see* Table 3.1). As an example, the evaluation result is given in Table 3.2.
8. Autoclave Erlenmeyer flasks with *in vitro* inoculated plantlets for decontamination (*see* Notes 6 and 7).

4 Notes

1. MIS medium is composed of half-strength MS macronutrients and MS micronutrients without a carbon source. Media commonly used for the growth and sporulation of Foc are carbohydrate-rich, and carbon has been shown to be the first limiting substrate of Foc growth in sterilized soil (Couteaudier and Alabouvette 1990). In this bioassay, the subtle balance between the growth of Foc and the carbon source is achieved by removing sucrose from the MS medium

and by employing a filter paper disc as carbon source. Since the rooted banana plantlets with leaves are able to photosynthesize, the use of MIS medium in this bioassay also guarantees the normal growth of the plantlets.

2. CAUTION: it is suggested to use a virulent strain of Foc TR4. The disease severity of *in vitro* inoculated plantlets is affected by several factors, such as the pathogenicity of the Foc TR4 strain, sporulation medium, components of MIS medium, and growing time of banana plantlet on MIS medium before *in vitro* inoculation. Among these, the pathogenicity of Foc TR4 strain is the most important factor (Wu et al. 2020). If variation in pathogenicity occurs, for example, due to repeated sub-culturing or long-term preservation in the lab, this bioassay is not useful to identify banana mutants which are resistant to Fusarium wilt.
3. CAUTION: not only plant material preparation but also inoculum preparation and inoculation of rooted banana plantlets are conducted under sterile conditions. In order to obtain reliable data of disease severity, it's necessary to acquire the ability of aseptic operation prior to the experiment.
4. Five plantlets of each cultivar are *in vitro* inoculated with Foc TR4, and the entire experiment is repeated three times ($n = 15$). Control plantlets are treated in the same manner except that filter paper discs are soaked in sterile distilled water.
5. For field evaluation and greenhouse bioassay, the resistance of banana cultivars tested is generally divided into five levels according to a disease index. Because the disease grades of *in vitro* bioassay are non-linear in disease severity, the numerical value obtained by substituting the disease index calculation formula cannot reflect the real disease severity. That is, when there is no quantitative limit for disease grade, the disease index calculation formula is not applicable. The logistic regression model belongs to a probabilistic nonlinear regression model (McCullagh 1980). Using Logistic regression to analyze the disease grade data greatly improves the accuracy of the *in vitro* bioassay.
6. The *in vitro* bioassay is a laboratory bioassay with effective containment conducted in a controlled environment. All experimental waste and materials must be autoclaved in order to prevent the spread of Fusarium wilt pathogen into the environment.
7. Since tissue-cultured banana plantlets get easily contaminated during plant material preparation and *in vitro* inoculation, it is necessary to re-isolate Foc TR4 by cutting a thin cross-section of the rhizome from a diseased plantlet followed by placing the disc directly onto a PDA plate. Then the cultures are identified by following the procedures described by Summerell et al. (2003).

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