Comparison of Methods for Inoculation of Ears and Stalks of Maize with *Fusarium moniliforme*

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ABSTRACT

Drepper, W. J., and Renfro, B. L. 1990. Comparison of methods for inoculation of ears and stalks of maize with *Fusarium moniliforme*. Plant Dis. 74:952-956.

Over four plantings from 1986 to 1988, several ear- and stalk-inoculation methods for *Fusarium moniliforme* were compared on maize (*Zea mays*). Significant differences were observed among methods in their effect on disease incidence and severity. For ear inoculations, shooting a BB pellet covered with inoculum into the ear or using a nail punch were the most effective methods. For stalk inoculations, shooting a BB pellet into the stem or the drill/toothpick method were the most effective. Ear and stalk rot severities were positively correlated with the diameter of the inoculation tool.

Ear and stalk rots are among the most destructive diseases of maize (*Zea mays* L.) in many areas of the world and cause large yield losses (1,3,7,8,18,19,22). The pathogens most frequently involved are *Fusarium* spp. (4,21,24). In addition to causing yield losses, *Fusarium* spp. can reduce grain quality by producing several mycotoxins (4,19) that adversely affect mammals (21). Wounds favor infection and development (21). The use of resistant varieties or hybrids to control the disease is more effective than the use of cultural measures or chemical treatments, which do not provide sufficient control (17).

Progress has been made in breeding for resistance to ear rot using artificial inoculation (8). Several ear-inoculation methods have been developed. Inserting an infested toothpick into the midear gives a higher rot rating than either placing the toothpick in the ear tip or shank or spraying an inoculum suspension onto the silks (10). Shooting a BB pellet into the ear was found to be superior to injecting a spore suspension into the tip of the ear with a hypodermic needle or spraying a spore suspension with a plastic squeeze bottle (5).

There are several reports in which stalk-inoculation methods were tested (6,7,16). Injection of a spore suspension and the insertion of infested toothpicks, wheat kernels, or agar disks were all found to be superior to the insertion of infested pipe cleaners (6). In other reports (1,10), good results were obtained with a nail punch, which combines drilling and inoculating simultaneously. There has been no general agreement as to which internodes of corn are the most susceptible to stalk rot. Some reports state an increase in susceptibility from the lower to the upper internodes and others state the opposite (7). Stresses from drought, mechanical wounds, high plant density, foliar diseases, and insects are reported to favor stalk rot (3,9,18).

The purpose of this study was to compare different ear- and stalk-inoculation methods for their effectiveness in creating high disease incidence and severity and to determine which methods were best for showing differences in resistance levels under conditions in Mexico.

MATERIALS AND METHODS

All trials were grown at CIMMYT experiment stations in Mexico from December 1986 until September 1988. Winter (December–April) trials were planted at Poza Rica and Tlaltizapan and summer (June–September) trials at those two sites and at El Batan. Poza Rica is a tropical station at 20° north latitude (elevation 60 m) with sandy loam soil, Tlaltizipan is a subtropical station at 18° north latitude (elevation 960 m) with predominantly clay soil, and El Batan is a highland station at 19° north latitude (elevation 2,249 m) with predominantly loam soil.

Tropical-subtropical maize germ plasm, consisting of five quality protein maize (QPM) S_1 lines with an endosperm possessing higher than normal protein quality and five S_1 lines with normal endosperm, were chosen for the first trials in the winter of 1986-1987. No significant differences in ear and stalk rot resistance were observed between the normal S_1 lines, so these were excluded and only three QPM lines were used for subsequent plantings.

A split-plot design with four replications was used, with inoculation methods as the main plot and cultivars as subplots. Each plot was a single row, 2.5 m long, and included 10 or 11 plants so that for each treatment a sample of 40 plants was available. Row spacing was 0.75 m and plant spacing was 0.25 m, which is equivalent to a plant density of 53,700 plants per hectare. A mixture of eight virulent isolates of *Fusarium moniliforme* Sheld. collected in Mexico was used as inoculum. Inoculum for the spore suspension was produced on sterilized oats in 1-L glass bottles. For the toothpick inoculation method, washed, autoclaved toothpicks were placed in small glass jars, moistened with 50 ml of potato-dextrose broth, and infested (7,26). The glass bottles and jars were kept at about 25 C and were radiated by fluorescent and incandescent lamps (with photon flux density of 20 and 60 $\mu E \cdot cm^{-2} \cdot s^{-1}$, respectively), for 10 hr daily. After 10–14 days under these conditions, colonies produced large quantities of conidia.

A hygrothermograph (Datapod 220, Omnidata International, Inc. Logan, UT) was installed at flowering time in the center of the trial area at each location. Temperature and relative humidity (RH) were recorded hourly from flowering until harvest when ear and stalk rot incidence and severity were evaluated. Inoculations were done 7–10 days after anthesis. Unless stated otherwise, the concentration of the spore suspension was adjusted to 1×10^6 conidia per milliliter in the 1987 plantings. In the winter of 1987-1988, the inoculum concentration was increased to 1×10^8 conidia per milliliter, but the concentration of the spore suspension was reduced to 1×10^7 conidia per milliliter for the 1988 summer planting.

Ear inoculation methods were compared in the winter 1986-1987 and in the summer 1987 plantings. Common methods used in both plantings included injecting 2 ml of the spore suspension into the silk channel; shooting an inocu-

Accepted for publication 13 March 1990 (submitted for electronic processing).

lum-covered BB pellet with a gas pistol into the midear (the BB pellets were washed with a detergent, rinsed in water, and placed over a petri dish overgrown with mycelium of *F. moniliforme* and shaken, so that approximately 1×10^6 spores became attached to the each BB pellet); inserting an infested toothpick into the midear; and punching the midear with the nail punch. The nail punch consisted of a 2.4-mm-diameter nail protruding 1.5 cm from a wooden handle. The nail, which had a 1-cm^3 piece of sponge at its base, was dipped into the spore suspension and punched into the ear. In the winter 1986-1987 planting, the method of inserting an infested toothpick into the silk channel was used, and in the summer 1987 planting, the method of punching the midear with spore suspensions of 1×10^6 and 1×10^7 spores per milliliter and water as a control were used.

After the 1987 results were analyzed, the nail punch method was employed in both experiments in 1988 with nails of different diameters (2.4 mm, 3.6 mm, 4.4 mm, and 5.3 mm). The method of injecting a spore suspension into the silk channel was also maintained, because this method has been the standard ear inoculation method at CIMMYT. In the winter 1987-1988 planting, the method of inserting an infested toothpick into the midear was used, while in the summer 1988 planting, the BB-pellet method was employed. For each planting, a control treatment was used where the plants were not inoculated. Disease incidence (percentage of diseased ears) and severity were measured on an individual plant basis at physiological maturity of the grain (black-layer stage). For severity, a rating scale of 1–5 was used, where 1 = no kernel infection, 2 = 1–10%, 3 = 11–20%, 4 = 21–30%, and 5 = 31% or more of the kernels infected.

For stalk inoculation, the middle of the first elongated internode was used, if not stated otherwise. Common methods tested in both plantings in 1987 included the 2.3-mm drill/toothpick method, nail punch/sponge method, nail punch and injection of 2 ml of a spore suspension, and the BB-pellet method. In the winter 1986-1987 planting, the method of drilling the stem and introducing an infested toothpick was also used on the internode below the ear. In the summer 1987 planting, the method of punching the stem with the nail punch and injecting 2 ml of a spore suspension were also used with two spore concentrations (1×10^6 and 1×10^7 spores per milliliter, respectively). In the same planting, the BB-pellet method was used with BB pellets without inoculum.

As a consequence of the 1987 results, the method of drilling and inserting an infested toothpick was employed in both experiments in 1988 with drill bits of different diameters. Drill bits with diameters of 2.3 mm, 3.5 mm, 4.7 mm, and 6.3 mm were used. In the winter 1987-1988 planting, the method of drilling a hole and injecting 2 ml of a spore suspension was used for drill bits 2.3 mm and 6.3 mm in diameter. Disease ratings (incidence and severity) were done at physiological maturity of grain (black-layer stage). For severity, plants were cut longitudinally and rated individually with the Hooker (14) scale, which indicates the percentage of infection in the inoculated internode: 1 = 0–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%, and 5 = 100%, with infection extending into an adjacent internode.

Data analysis employed analysis of variance and regression. In the analysis of variance, the F test was used to test the hypothesis of all means being equal and the level of significance attained as presented in the results section. Duncan's multiple range test was then applied to assess significance of the differences between pairs of means at the 5% level.

RESULTS

Because no significant cultivar × method or method × location effects were observed, disease data on all cultivars across locations were used for the analysis. Ear rot severities and incidences resulting from the inoculation methods were significantly ($P = 0.01$) higher (except for the infested toothpick inserted into the silk channel in 1986-1987) than the noninoculated control treatments in the winter planting of 1986-1987 and the summer planting of 1987 (Table 1). This was true for each individual planting as well as for the analysis over both plantings. The nonwounding method used in the winter 1986-1987 planting of inserting a toothpick into the silk channel resulted in significantly lower disease severity than the wounding-type inoculation methods (BB pellet, toothpick/midear, and nail punch). The syringe method did not differ significantly from the toothpick/silk channel method and is not regarded as a nonwound method, as the cob is often wounded by needle insertion.

The BB-pellet method caused significantly higher ear rot severity and incidence than the nail punch, toothpick/mid-ear, and syringe methods, which did not differ significantly from one another. In the winter of 1986-1987, the insertion of an infested toothpick into the silk channel was the least effective inoculation method. It did not differ significantly from the toothpick inserted into the silk channel nor from the noninoculated control and was thus discontinued.

Data from both inoculation trials in 1987 indicated that ear rot severity may be influenced by the size of the wound. Disease data from the BB pellet (4.6 mm in diameter), nail punch (2.4 mm), toothpick (2.1 mm), and the noninoculated treatment were regressed against diameter for the winter and the summer plantings separately, and significant positive correlation coefficients ($P = 0.01$) were obtained with R^2s of 0.70 and 0.71, respectively. Therefore, the inoculation methods were changed for the trials in 1988, where nail punches with nails of different diameters were used for inoculations.

In the winter planting of 1987-1988, all inoculation methods resulted in significantly ($P = 0.01$) higher disease severity and incidence than did natural infection (Table 2). The syringe method caused lower disease severity than nail punches of the three largest sizes. No significant differences, however, were found between nail punches of different sizes and the toothpick/midear method.

In the summer 1988 planting (Table 2), few significant differences between inoculation methods were detected. Disease data across locations were influenced by extensive lodging in Poza Rica. Many ears had been in contact with the soil for 2 days before inoculation and natural infection levels were high, as evidenced by the elevated severity ratings for the control. Differences in ear rot caused by the inoculation methods were therefore masked by high natural infection. However, analysis for the separate locations in summer 1988 showed that at Tlaltizapan, the BB-pellet method and

Table 1. Disease severity (DS) and disease incidence (DI) of maize ear rot after different inoculation methods for *Fusarium moniliforme* for two plantings in 1987

Inoculation method	Winter 1986-1987		Summer 1987		Mean 1987	
	DS	DI (%)	DS	DI (%)	DS	DI (%)
BB pellet	2.42 a[x]	78 a	3.16 a	91 a	2.79 a	85 a
Toothpick midear	1.88 b	62 b	2.39 b	73 b	2.14 b	68 b
Punch + 10^7 ml	...[y]	...	2.36 b	74 b	...	...
Punch + 10^6 ml	1.92 b	60 b	2.19 b	70 b	2.05 b	65 b
Punch + water	...	...	2.17 b	68 b	...	...
Syringe s.c.[z]	1.80 bc	53 bc	2.24 b	68 b	2.02 b	61 b
Toothpick s.c.	1.67 cd	46 cd	...	...	...	...
Control	1.56 d	42 d	1.83 c	49 c	1.70 c	46 c

[x] Means over germ plasm and locations. Means within the same column followed by the same letter are not significantly different from each other according to Duncan's multiple range ($P = 0.05$).

[y] Inoculation method not used that planting.

[z] s.c. = silk channel.

the 4.4-mm nail punch resulted in significantly more disease than the other methods.

Of the genotypes in the winter planting of 1986-1987, the QPM lines were significantly more susceptible ($P = 0.01$) than the normal endosperm lines. The rankings for ear rot resistance of the cultivars used in the trials were about the same for all inoculation methods. No data on the resistance rankings, however, are presented here because of the large number of inoculation methods, plantings, and cultivars. In summer plantings, more disease developed and more differences in ear rot resistance between cultivars were detected than in winter plantings. With natural infection, disease severity was lower than in the inoculated treatments and fewer differences were found in the resistance rankings of the cultivars. Disease was more uniform in the uninoculated control treatment than in the inoculated treatments. For our experiments, to rate an accession with a precision of L = −0.25 on a 1-5 scale at a confidence level of $P = 0.05$ using the formula $n = 4s^2/L^2$ (s = standard deviation), a sample of $n = 70$ plants would be needed in the inoculated treatments, while 48 plants would suffice in the noninoculated treatment.

In the winter 1986-1987 planting and the summer 1987 planting, the stalk inoculation methods resulted in significant differences in disease incidence and severity ($P = 0.01$) for each planting as well as across plantings (Table 3). Incidence and severity were higher in inoculated treatments than in the control treatment, which was left to natural infection. The BB-pellet method was the most effective in causing high disease incidence and severity. Following in order of efficiency were the nail punch/injection and drill/toothpick methods. In the winter planting, no difference was observed between the last two, whereas in the summer planting, the nail punch/injection method caused more disease than the drill/toothpick method. Inoculating the internode below the ear with the drill/toothpick method was less efficient than inoculating the first elongated internode.

The nail punch/injection technique was significantly more efficient than the nail punch/sponge method, which inserted less inoculum into the wound. In contrast, using BB pellets with or without inoculum (relying only on natural infection in the latter case) produced equal disease incidence and severity. The nails and the pellets had respective diameters of 2.4 mm and 4.7 mm.

Regression analysis for each planting using inoculation tool diameter and stalk rot severity gave highly significant correlation coefficients ($P = 0.01$), with R^2 values greater than 0.78. For this reason, in the 1988 inoculations the drill/toothpick method was employed with different bit diameters.

Significant differences in stalk rot severity and incidence were found among the inoculation methods employed in the winter 1987-1988 planting ($P = 0.01$), for the summer planting ($P = 0.01$), and between identical methods over both plantings (Table 4). Disease incidence and severity was significantly higher in all inoculation treatments than in control treatments. The largest drill bit (6.3 mm in diameter) produced significantly higher severity than the smaller bits. No significant difference was found between the drill method combined with a toothpick or a spore suspension.

The 4.6-mm-diameter BB pellet caused disease incidence and severity levels similar to those produced by the 4.7-mm bit with a toothpick in the summer planting. This again illustrates the importance of tool diameter in artificial stalk inoculation.

Stalk rot incidence was always very low with natural infection, ranging from 8 to 25% in the four plantings. Most of the inoculation methods resulted in disease incidence levels of 75–100%.

Table 2. Disease severity (DS) and disease incidence (DI) of maize ear rot after different inoculation methods for *Fusarium moniliforme* for two plantings in 1988

Inoculation method	Winter 1987-1988		Summer 1988		Mean 1988	
	DS	DI (%)	DS	DI (%)	DS	DI (%)
BB pellet	...[x]	...	2.33 a	78 a	...	...
Punch (5.3 mm)	2.30 a[y]	76 ab	2.27 ab	76 a	2.29 a	76 a
Punch (4.4 mm)	2.28 a	82 a	2.23 ab	62 ab	2.26 a	72 a
Punch (3.6 mm)	2.32 a	80 ab	2.32 a	61 ab	2.32 a	71 ab
Punch (2.4 mm)	2.27 ab	78 ab	2.13 b	52 b	2.20 a	65 ab
Toothpick midear	2.22 ab	74 ab	...	...	...	...
Syringe s.c.[z]	2.11 b	70 b	2.25 ab	75 ab	2.18 ab	73 a
Control	1.81 c	55 c	2.16 ab	62 ab	1.99 b	59 b

[x] Inoculation method not used that planting.
[y] Means over germ plasm and locations. Means within the same column followed by the same letter are not significantly different from each other according to Duncan's multiple range ($P = 0.05$).
[z] s.c. = silk channel.

Table 3. Disease severity (DS) and disease incidence (DI) of maize stalk rot after different inoculation methods for *Fusarium moniliforme* for two plantings in 1987

Inoculation method	Winter 1986-1987		Summer 1987		Mean 1987	
	DS	DI (%)	DS	DI (%)	DS	DI (%)
BB + inoculum	3.52 a[y]	100 a	3.64 a	99 a	3.58 a	99 a
BB, no inoculum	...[z]	...	3.58 a	98 a	...	...
Drill/toothpick	2.83 b	92 ab	2.61 c	75 b	2.72 bc	87 b
Drill, high internode	2.41 c	83 cd	...	...	...	...
Punch + 10^7 ml	...	...	3.32 b	93 a	...	...
Punch + 10^6/ml	2.67 b	89 bc	3.31 b	90 a	2.99 b	89 b
Punch/sponge	2.32 c	77 d	2.64 c	78 b	2.48 c	77 c
Control	1.28 d	19 e	1.46 d	25 c	1.37 d	22 d

[y] Means over germ plasm and locations. Means within the same column followed by the same letter are not significantly different from each other according to Duncan's multiple range ($P = 0.05$).
[z] Inoculation method not used that planting.

Table 4. Disease severity (DS) and disease incidence (DI) of maize stalk rot after different inoculation methods for *Fusarium moniliforme* for two plantings in 1988

Inoculation method	Winter 1987-1988		Summer 1988		Mean 1988	
	DS	DI (%)	DS	DI (%)	DS	DI (%)
Drill (6.3) + 2 ml[x]	3.39 a[y]	100 a	...[z]	...	...	...
Drill (6.3) + toothpick	3.31 a	99 a	3.46 a	83 a	3.37 a	91 a
Drill (4.7) + toothpick	3.12 b	100 a	3.09 b	77 ab	3.11 b	89 a
Drill (3.5) + toothpick	3.05 bc	98 a	3.01 b	77 ab	3.03 b	88 a
Drill (2.3) + toothpick	3.00 bc	95 a	2.94 bc	77 ab	2.97 b	86 a
Drill (2.3) + toothpick	2.92 c	91 a	...	...	...	...
Drill (2.3) + H_2O	...	...	2.80 c	66 b	...	...
BB pellet	...	...	3.07 b	78 ab	...	...
Control	1.47 d	15 b	1.23 d	8 c	1.35 c	12 b

[x] Injection of 2 ml of spore suspension.
[y] Means over germ plasm and locations. Means within the same column followed by the same letter are not significantly different from each other according to Duncan's multiple range ($P = 0.05$).
[z] Inoculation method not used that planting.

Stalk rot severity was fairly uniform among treatments. Under the climatic conditions prevailing at CIMMYT's experiment stations, to rate an S_1 line with a precision of 0.25 on the 1–5 scale at 95% confidence, a sample of 40 plants would be needed relying on natural infection. For the BB-pellet method, the sample size would be 42 plants, for the nail punch/sponge method 68 plants, and for the nail punch/injection method 64 plants. Using the drill/toothpick method, uniformity increased with drill size, so with 2.3-mm, 3.5-mm, 4.7-mm, and 6.3-mm drill bits, the same precision in rating an S_1 line required 43, 36, 26, and 27 plants, respectively.

DISCUSSION

Koehler (16) reported wounding types of inoculation caused more ear rot than nonwounding types. Boling et al (5) found the BB-pellet method to be the most efficient. Our experiments confirm these findings. We obtained the highest disease severity with the BB-pellet and nail-punch methods, followed by the toothpick/midear and the spore suspension injection methods. The nonwounding method of inserting a toothpick into the silk channel was less effective than the inoculation methods requiring wounding. This is also shown by several studies that demonstrated the superiority of the method of sticking an infested toothpick into the midear over the nonwounding method of spraying a spore suspension onto the silks (10,12). Reports of increased incidence of *F. moniliforme* after attack by different insects (21) also indirectly support the findings of the effectiveness of the wounding inoculation methods. The insects damage the kernels and can carry the pathogen directly into the kernels or facilitate its entry. There are no reports on the influence of mechanical wound size on disease severity, which was found to be significant in our experiments.

Gulya et al (12) got high disease severity and good discrimination between resistant and susceptible germ plasm with the toothpick/midear method. Warren (25) obtained high incidence of infection and a clear differentiation among genotypes when silks were spray-inoculated 6–18 days after pollination. Ullstrup (23) found that spraying the tip of the ears with *Gibberella zeae* (Schwein.) Petch and covering them with wet paper towels and a water-repellent cap was better for differentiating between resistant and susceptible genotypes than injecting a spore suspension or inserting an infested toothpick into the silk channel, because the latter methods caused too much rot. When disease incidence was at a range of 80–100% or disease severity was at a range of 29–97%, he did not obtain a clear differentiation between genotypes. In our study, injection of a spore suspension or insertion of a toothpick into the silk channel caused relatively low rot ratings and low incidence. Therefore, the high disease development in experiments by Ullstrup (23) and Warren (25) was probably caused by highly favorable climatic conditions for rot development. As the environment at CIMMYT's experiment stations in Mexico is often unfavorable for Fusarium ear rot development, the wound inoculation methods are better suited at these stations, as they result in higher disease levels. Because the susceptibility ranking of materials and the needed sample size for ranking a material with a desired precision was the same for all inoculation methods, the 3.6-mm nail punch was adopted for CIMMYT ear inoculations. This method resulted in the highest disease severity permitting better differentiation between resistant and susceptible germ plasm (*unpublished*).

The highest stalk rot levels in our experiments were produced by the use of BB pellets with or without inoculum, indicating that the size of the wound may be more important than the amount of inoculum introduced. This conclusion is supported by the fact that the BB pellet without inoculum still resulted in greater rot severity than inoculation tools of a smaller diameter with inoculum. The reason for this may be that the pathogen more easily invades larger wounds caused by inoculation tools of a greater diameter. However, the quantity of inoculum introduced has importance, because the nail punch method was more efficient when greater amounts of inoculum were injected than the nail punch/sponge method, which only introduced a small amount of the spore suspension contained in the 1-cm^3 sponge. The amount of inoculum may thus be more important with smaller wounds.

The relationship between wound diameter and stalk rot severity was found to be highly significant in our experiments. No previous work has reported similar results. Cappellini (6) stated that introducing inoculum of *G. zeae* with infested pipe cleaners was inferior to infested toothpicks, wheat kernels, agar disks, and spore suspensions. However, he reported no difference in stalk rot production between the use of a 3-mm hypodermic syringe, a 7-mm cork borer, or an icepick (diameter unspecified). Because the inoculum used in Cappellini's work was grown on different media for the different techniques, the effect of tool diameter on stalk rot levels is difficult to evaluate.

Lower disease incidence and severity were obtained in our experiments by inoculating the internode below the ear than by inoculating the first elongated internode. These results support the report in which less stalk rot was observed at higher internodes (7).

The method developed by Koehler and Young (16,26) of drilling (2.4-mm drill bit) and inserting an infested toothpick is being maintained for CIMMYT stalk inoculations. The use of larger drill bits would increase disease severity and uniformity, but it would also increase the chance of infection by other pathogens at the site of the wound, an effect which may not be desirable.

For evaluating the efficiency of ear and stalk inoculation methods, rating disease severity seems to be a more useful parameter than rating disease incidence, as incidence differed little among methods, whereas with severity more significant differences were expressed.

Nonwounding inoculation methods closely simulate natural infection. Therefore, selecting natural infection or using nonwounding methods contributes more to resistance based on morphological traits, assuming that insects are controlled. Wound-type inoculations simulate insect attack to some degree as they bypass morphological barriers. In the case of ear inoculation, disease development on wounded kernels is related to the physiological resistance of the kernel tissue (11). The further spread to noninjured kernels depends again on the morphological resistance. Therefore, when selecting for ear rot resistance, after wound inoculations both morphological and physiological traits are involved.

In breeding for stalk rot resistance, progress was reported with artificial inoculation (20). Although natural infection and inoculation do not always give the same result (13), investigators have reported statistically significant correlations between the two screening methods (3,16,20). Barnes (2), however, questioned the reliability of inoculation, as in several instances he found a lack of correlation between natural and artificial infections that indicated that breeding programs should also employ selection under natural infection. Prolonged wet weather has been reported to promote ear damage during the grain development stage (15), as well as during periods of warm weather with persistent wetness (21).

The change of spore suspension concentration in the two 1988 plantings could have affected disease severity to some degree. However, the results of the 1987 summer planting show that spore suspensions of different densities did not significantly affect disease severity and therefore the change of the spore suspension in 1988 probably exerted only a minor effect on disease severity.

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