Influence of Inoculum Preparation and Volume on Growth of Mycotoxicogenic Molds

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ABSTRACT

The influence of the volume and the $a_w$ of the inoculum, adjusted or not with glycerol to the $a_w$ of the medium, on growth of mycotoxicogenic species of fungi was determined for Aspergillus flavus, Aspergillus ochraceus, Penicillium citrinum, Penicillium viridicatum, Fusarium tricinctum and Microdochium nivale.

Statistical analysis of the data was based on the application of multivariate techniques.

It was seen that at constant volumes (10 μl), P. viridicatum, A. ochraceus and M. nivale show no significant growth differences whether or not the $a_w$ of the inoculum is adjusted; but significant differences were observed with A. flavus, P. citrinum and F. tricinctum. Moreover, significant differences in growth between adjusted and unadjusted $a_w$ levels are also present when there are different inoculum volumes for A. flavus and P. citrinum. Thus, it is appropriate to adjust the inoculum to the same $a_w$, level as that of the medium or the food to be considered. In all cases, where significant differences were present, greater colony diameters were observed when the $a_w$ was adjusted.

With the same inoculum preparation, different inoculum volumes also affect fungus growth, producing greater colony diameters with increments in the volume.

Key words: Mycotoxicogenic fungi, inoculum volume, water activity

When growth of toxigenic fungi is analyzed, many sources of variability should be taken into account (14). It is necessary to consider that what is estimated to be biological variation may be due to a lack of standardization of methodologies, and therefore results obtained by various scientists are very difficult to compare.

Among other difficulties, there is a lack of information on the methodology utilized in the preparation of inocula and on standardization in terms of temperature and time (a majority of papers indicate temperatures between 25°C and 30°C and a period of 4 to 10 days [3,4,14,17]). Sometimes the number of spores per milliliter is reported, but neither the volume of inocula nor the value of the water activity, $a_w$, are specified. Among other things, uncertainty in the measurement of the number of spores (13) can be observed.

The objective of this paper is to determine the influence of inoculum volume and the adjustment of the $a_w$ of the inoculum on the growth of colonies of toxigenic fungi in solid media, since most of the papers deal with dry spores (8,18) which are not the forms present during storage. The concentrations of spores used were similar to those normally found in field contamination and during storage of grains (16).

It was decided to use agar media in order to identify clearly the effects of the volume size and $a_w$ of the inocula, isolating them from other sources of variability.

Furthermore it was considered essential, because of the uncertainty of the lag-phase estimation, to develop statistical tools to determine fungal behavior with higher precision, considering all aspects of the behavior of the growth.

MATERIALS AND METHODS

Fungi and media

Aspergillus flavus Link and Penicillium citrinum Thom, two strains of each, were isolated from samples of corn collected in the Province of Buenos Aires, Argentina. To complete the study, one strain of Penicillium viridicatum Westling and one of Microdochium nivale (Fr.) Samuels and Hallett came from the culture of the Food Microbiology Laboratory, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, as well as Aspergillus ochraceus Wilhelm NRRL 3177 and Fusarium tricinctum (Corda) Sacc. NRRL 24631.

Identification of isolates was carried out in our laboratory according to Pitt and Hocking (9), Samson and Pitt (11), and Nelson, Toussoun, and Marasas (7).

Malt extract agar (Merck, Cat. No. 5398) was used as the basal medium with an $a_w$ of 0.999 (2). Amounts of glycerol (Merck, Cat. No. 4094) added to the basal medium to produce different $a_w$ levels were calculated according to the studies of Chirife, Ferro Fontán, and Benmergui (1).

Water activity

Measurements were made at 25°C using an electronic hygrometer Vaisala Humicap HMI33 (Vaisala Oy, Finland), with a sorption probe Sonde DK 159 (Driesen and Kern, F.R.O.) calibrated with saturated salt solutions (10).

Inoculum preparation

Fungi were subcultured in potato dextrose agar (Merck, Cat. No. 10130) slants from the stock cultures.
Strains were incubated for 7 days at 25°C and harvested with 5 ml of a solution of 0.06 g/l of Tween 80 (Merck, Cat. No. 822187) in water for unadjusted inocula (6). In order to obtain inocula adjusted to an a_w of 0.850, 0.900, 0.925, 0.950, and 0.970, glycerol was added to the former solution. Glycerol was selected, following Pitt and Hocking (8), who recommended it in preference to NaCl because it does not inhibit growth.

The concentration of spores, measured with a hemacytometer, was on the order of 10^7 spores per milliliter. Dilutions were made in order to place 10^4 spores on each Petri dish in all the cases under study and were measured again before inoculation.

**Inoculation and incubation**

Dishes were inoculated slowly with micropipettes of variable volume in order to obtain a circular inoculum in the middle of the plate and to avoid spreading.

For each species studied, growth in diameter of the colonies was determined using 5 replicates.

Plates were incubated at 25°C for various times, inside of polyethylene bags of 40 μm thickness, in order to avoid variations in a_w. Assays were distributed into two groups:

a. **Variation in a_w of the inoculum at constant volume**. Spores of pure cultures of A. flavus, P. citrinum and F. tricinctum were placed on media at levels of a_w 0.900, 0.950, and 0.970, and P. viridicatum, A. ochraceus, and M. nivale on media with a_w of 0.850, 0.900, 0.925, 0.950, and 0.970. In all cases, the volume of the inoculum was 10 μl. All the experiments were done twice, once with adjusted inocula and once with unadjusted inocula.

b. **Variation of volume of the inoculum, with adjusted and unadjusted a_w**. Inocula of 1, 5, 10, and 25 μl of A. flavus and P. citrinum were cultured on media with an a_w of 0.900. P. citrinum was also cultured on media with an a_w of 0.850. As in the case of assay a, all the experiments were done with both adjusted and unadjusted inocula.

**Growth measurements**

Average diameters of colonies were recorded in millimeters. These averages were computed from two measurements at right angles with each other, from 5 replicate colonies (9).

**Statistical methods**

The influence of the volume of the inoculum and of the addition of glycerol as a solute to adjust the a_w, was established by use of multivariate techniques which allow an analysis of vectors of correlated random variables. In this case, the repeated measurement through time of a colony’s diameter is the vector of dependent variables. The hypothesis tested was that the vector of means from the different populations were equal. Each population is defined in terms of the values taken by the variables considered in this research (volume of inoculum and glycerol).

The technique used to compare different populations’ means is the multivariate analysis of variance (12). This analysis can be carried out when the following two assumptions are fulfilled: The first states the equality of variance and covariance matrices in different populations. The other assumes the multivariate normal distribution of measurement errors. The first hypothesis was validated using Cochran’s C test, the F tests of Bartlett-Box, and the multivariate Box M test. To validate the normality of the errors, univariate boxplots were designed, which only validate the normality of each variable and not the joint normality.

The usual statistics in multivariate analysis (Pillai, Wilks lambda, Hotelling and Roy) were used to test the hypothesis of equality of means. When the hypothesis of equality of the variance and covariance matrices of the two populations was rejected, the James modification was used. This modification allows a test of approximate level, based on the χ² distribution.

In order to obtain the four statistics mentioned, the univariate tests for variance and the boxplots, the MANOVA procedure of package SPSS (15) was used. In other cases, software specially developed for this analysis was used.

**RESULTS**

The analysis of the influence of the volume of inocula and the glycerol adjustment of the a_w, by means of the Pillai, Wilks lambda, and Hotelling and Roy tests, lead to the same conclusions. That is why only the results of Pillai’s test are presented. On the other hand this is the most robust one, in the sense that its significance level is only slightly modified when the underlying assumptions are violated. Under normality all four tests have similar power and for small deviations from this assumption, Pillai’s test is powerful.

In those cases in which the Box test rejects the hypothesis of equality of covariance matrices, the statistic used was James’s; the tests used are indicated on the tables.

### Table 1. Influence of a_w adjustment of inocula volume (10 μl).

<table>
<thead>
<tr>
<th>Fungi</th>
<th>a_w</th>
<th>0.85</th>
<th>0.90</th>
<th>0.925</th>
<th>0.95</th>
<th>0.97</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td></td>
<td>*</td>
<td>5.4</td>
<td>291.4</td>
<td>14.87</td>
<td>8.73</td>
</tr>
<tr>
<td>P. viridicatum</td>
<td>163</td>
<td>336</td>
<td>96.94</td>
<td>0.70</td>
<td>25.80</td>
<td></td>
</tr>
<tr>
<td>A. ochraceus</td>
<td>81</td>
<td>8.1</td>
<td>8.1</td>
<td>7.2</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>P. citrinum</td>
<td>42.5</td>
<td>3.97</td>
<td>171.16</td>
<td>13.62</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>M. nivale</td>
<td>8.1</td>
<td>8.1</td>
<td>8.1</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>F. tricinctum</td>
<td>0.118</td>
<td>0.371</td>
<td>0.059</td>
<td>0.028</td>
<td>0.551</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fungi</th>
<th>a_w</th>
<th>2,7</th>
<th>5.4</th>
<th>4.5</th>
<th>0.000</th>
<th>0.000</th>
<th>0.000</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. citrinum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.7</td>
<td>5.4</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>M. nivale</td>
<td>4.05</td>
<td>3.38</td>
<td>1.44</td>
<td>8.88</td>
<td>2.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.367</td>
<td>0.398</td>
<td>0.571</td>
</tr>
<tr>
<td>F. tricinctum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>571.91</td>
<td>93.78</td>
<td>241.35</td>
</tr>
</tbody>
</table>

* a_w not considered
  1Approximate F-statistic
  2Degrees of freedom
  3F significance

**Table 1. Influence of a_w adjustment of inocula volume (10 μl).** Pillai’s test of significance for detecting differences between adjusted and unadjusted a_w.
lyzed for different inoculum volumes with an \( a_w \) level of 0.900 for \( A. \) flavus and 0.850 and 0.900 for \( P. \) citrinum. Table 2 shows that independently of the size of inoculum volumes, mean colony diameters still differ significantly with the \( a_w \) of the inoculum.

**b. Variation of both inoculum volume and \( a_w \)**

In Table 3, the results are presented corresponding to a comparison of inoculum volumes of 1, 5, 10, and 25 \( \mu l \) for \( A. \) flavus and \( P. \) citrinum with \( a_w \) adjusted or unadjusted. In all the cases under study, significant differences in growth were detected depending on the volumes inoculated, but in the multiple-comparisons procedure, no significant differences between 5 and 10 \( \mu l \) inocula were present.

It was of interest to test the hypothesis that the mean of the colony diameter increases with inoculum volume for each observation time for \( P. \) citrinum. Figure 2 presents a plot of mean colony diameter versus incubation time at the different volumes studied, with the inoculum adjusted to an \( a_w \) of 0.900.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Volume (( \mu l ))</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1026.86** 2257.18** 2399.22** 163807.20*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( A. ) flavus</td>
<td>6.2 6 5 6</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>41.46** 20.62** 1760.75** 1102.40*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P. ) citrinum</td>
<td>4.5 4.5 5.4 6</td>
<td>0.001</td>
<td>0.003</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*James (approximate chi square)
**Pillai (approximate F)

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Volume (( \mu l ))</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>214.00** 950.91** 8489.24** 1725.55*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P. ) citrinum</td>
<td>3.6 2.7 5 4</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume (( \mu l ))</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36, 0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.46, 0.66</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.60, 0.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.96, 1.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.97, 2.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.40, 2.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Confidence intervals of level 0.995 for mean growth of \( P. \) citrinum at each volume and time (\( a_w = 0.90 \), adjusted).

It was not possible to test the hypothesis of equality between means using a two-way analysis of variance, since the estimated variances in many cases are equal to zero. In order to test the proposed hypothesis, confidence intervals of level 0.995 were designed for each fixed volume and time. In some cases the corresponding intervals are reduced to a single point, since the estimated variances are zero, as noted. It was observed that the intervals obtained are never overlapping. This allows us to state that the mean colony diameter increases when passing from a volume of 1 to 5 \( \mu l \) and from the 5 to 25 \( \mu l \) with a significance level of 0.105.
CONCLUSIONS

These experiments suggest that the preparation of an inoculum, as well as the volume used for inoculation, influence fungus growth. In order to obtain comparable results when studying the behavior of toxigenic fungi, it seems necessary to standardize the inoculum preparation and application.

Since different behaviors were detected at all inocula volumes studied, whether glycerol was added or not, it is recommended that the $a_w$ of the inoculum be adjusted to the $a_w$ level of the medium or the food, to avoid problems during the equilibration time.

Also, inoculum volume should be taken into account, since it influences fungus growth, increments in volume producing greater colony diameters. It is suitable to use a volume in the range of 5 to 10 $\mu l$ in preparations, because no significant differences between these two volumes were detected.

ACKNOWLEDGMENTS

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