ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLIV, nr 3
1975

Effectiveness of different methods of preparing seedlings of cereals under sterile conditions for investigations of nucleic acids using radioactive precursors

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(Received: December 27, 1974)

Abstract

On seedlings grown from grain not sterilized in advance bacteria numbered more than 10^8 microorganisms per one gram of fresh weight. Washing of seedlings with cetyltrimethylammonium bromide or sodium lauryl sulphate solutions reduced the number of bacteria several times. Sterilization of grains prior to planting with sodium hypochlorite and germination on solid substratum (perlit) reduced the number of bacteria to below 10^4 microorganisms to one gram of fresh weight. If germination was, however, in water, bacteria on the seedlings were approximately as numerous as on seedlings from non-sterilized grains. Of the three antibiotics tested (streptomycin, chloramphenicol, penicillin) the most effective against the bacteria of wheat seedlings was chloramphenicol coupled with streptomycin; of antibiotics used singly chloramphenicol was best.

INTRODUCTION

The considerable effect of contamination with bacteria on the results of studies on the incorporation of radioactive precursors into nucleic acids in plants has been demonstrated by: Lonberg-Holm (1967), Hock (1967), Burdett and Wareing (1968) and Bendich (1972). Though the studies of Sobota et al. (1968) have shown that in some cases this effect may not be pronounced, the sterility of the plant material in such studies is in the evaluation of the correctness of the followed procedures, regarded, however, as a very important criterion. This is why these studies as a rule employ various treatments ensuring proper conditions of sterility. These primarily include treatment of the grains with various bactericidal agents with subsequent cultivation of the plants in sterile conditions. Only some studies have employed treatment of plants just prior to the use of

radioactive precursors (Burdett and Wareing, 1968; Aspart-Pascot et al., 1973; Callow and Woolhouse, 1973; Watanable and Imaseki, 1973). The effect of the bacteria is also commonly inhibited by antibiotics. These are added to the incubation environment either together with the radioactive precursors or somewhat earlier (Basler, 1966; Lonberg-Holm, 1967; Walton et al., 1970) in order to inhibit the growth of the bacteria, this being tantamount to the elimination of their influence. The antibiotics are employed in concentrations regarded as not bearing on the processes studied in the plant material (Parthier, 1965; Venis, 1967; Sood and Pillay, 1972). The most widely used sterilizing agents are solutions of sodium hypochlorite (Burdett and Wareing, 1968; Johri and Varner, 1970) and calcium hypochlorite (Callow and Woolhouse, 1973) and of the antibiotics chloramphenicol (Sobota et al., 1968; Walton and Soofi, 1969; Johri and Varner, 1970; Walton et al., 1970; Watanabe and Imaseki, 1973), streptomycin (Cherry and Huystee, 1965; Ingle and Key, 1965; Walbot, 1973) and penicillin (Kulaeva et al., 1971; Selivankina et al., 1972) and in some cases their combinations (Basler, 1966; Sood and Pillay, 1972). The use of detergents for the sterilization of plant material has also been attempted: Patterson and Smillie (1971) employed cetyltrimethylammonium bromide for the sterilization of wheat leaves and Aspart-Pascot et al. (1973) used sodium lauryl sulphate for the sterilization of *Raphanus* seedlings. The effectiveness of the presented methods is not sufficiently investigated and in many cases has not been confirmed. The purpose of our studies was to campare some of the methods with respect to their efectiveness in ensuring the sterility of seedlings grown from grain, thus enabling studies on the incorporation of radioactive precursors into their nucleic acids. The necessity of ensuring proper sterility in such studies has been shown by our investigations on the incorporation of radioactive phosphorus into the nucleic acids of not sterilized wheat seedlings and wheat seedlings cultivated in sterile conditions (unpublished data). The chromatographic profiles of the nucleic acids from these plants obtained as a result of chromatography on MAK columns have been found to differ considerably with respect to the distribution of radioactivity and were similarly dependent on the presence of bacteria in the investigated material as the chromatographic profiles of the nucleic acids from sterile and not sterile water melon seedlings, presented in the paper by Hock (1967).

MATERIAL AND METHODS

The studies were conducted on 'Ostka Złotokłosa' winter wheat seedlings grown from both sterilized and not sterilized grains. Several experiments were also conducted with 'Ostka Chłopicka' spring wheat seedlings

grown from grain not sterilized in advance. Sterilization was performed as follows: the grains were washed for 2 minutes with 96 per cent ethanol (Ryka, 1963), treated for 5 minutes with a 5 per cent solution of sodium hypochlorite and then washed with several changes of sterile water. Germination was maintained for 4 days in the dark at 22°. Some of the seedlings grown from grain not sterilized in advance were washed first for 15 minutes with either a 0.1 per cent solution of cetyltrimethylammonium bromide (Patterson and Smillie, 1971) or 2 per cent solution of sodium lauryl sulphate (Aspart-Fascot et al., 1973) and then with sterile water.

The number of bacteria on the seedlings as well as the sensivity of the bacteria to the employed antibiotics was estimated on the basis of the number of colonies after inoculation on agar medium by the plate method. The composition of the medium (in grams) was: peptone tryptone (Difco) — 5, yeast extract (Difco) — 2.5, agar — 15, glucose — 1, H_2O — 1000. The medium was supplemented with various antibiotics according to the diagram presented in the table containing the results. After inoculation the Petri plates were placed in thermostat for 72 hours at 30° and the number of colonies scored.

RESULTS AND DISCUSSION

The results in the table show the number of bacteria on wheat seedlings grown from grain not sterilized in advance to reach several hundred millions per one gram of fresh weight. A similar degree of contamination with bacteria was determined by Lonberg-Holm (1967) for lettuce and raphanus seedlings. The author also demonstrated that the results of studies on the labeling of the nuclei acids of plants with ^{32}P can be affected by the bacterial content in the plant material of the order of even 10^4 microorganisms per one gram of fresh weight. This indicates that as acceptable can be regarded only those methods for the preparation of the plant material resulting in not more than a few thousand bacteria per one gram of fresh weight thus compelling the use of treatment furthering the efficient elimination of the influence of the bacteria. An over ten-fold reduction in the number of bacteria — as shown in the table — can be achieved by washing the wheat seedlings with a solution of CTA-Br or SLS. The obtained results suggest the considerable usefulness of these agents for the reduction of the number of bacteria in plant material. However, the effect of these agents on plant material has not yet been investigated.

The results illustrating the sensivity of the bacteria on wheat seedlings to the employed antibiotics prove the most effective bactericidal action to be achieved by chloramphenicol coupled with streptomycin. Of the

singly used antibiotics chloramphenicol was best. The addition of penicillin to a combination of chloramphenicol and streptomycin (Table, items 11 and 14) resulted in but a slight increase in the bactericidal action. Similar dependences were also observed during studies on 'Ostka Chłopicka' spring wheat seedlings showing similar number of bacteria. The combining of various antibiotics enables the achievement of antibacterial spectra best suited for the bacteria on a particular biological material (Laskey, 1970; Watts and King, 1973). The above data show that in our case the most useful against the bacteria on wheat seedlings is a combination of streptomycin and chloramphenicol. However, as the composition of the bacterial flora in the same material is subject of changes it seems advisable to check the effectiveness of the antibacterial action of the antibiotics used in individual experiments.

The results relating to the antibacterial action of streptomycin and chloramphenicol used each at concentration 100 mg/l indicate that a combination of these antibiotics during the incubation of wheat seedlings not sterilized in advance in solutions containing radioactive precursors of nucleic acids allows for about 10^5—10^6 microorganisms not sensitive to these antibiotics per one gram of fresh weight. Treatment of the seedlings with CTA-Br or SLS reduces the number of bacteria to about 10^4—10^5. This means that in the case of wheat seedlings grown from grain not sterilized in advance washing with detergents and use of the antibiotics mentioned in this paper does not ensure the reduction of the number of active bacteria to below 10^4 per one gram fresh weight of the seedlings. Seedlings with number of bacteria reduced below this value were obtained with sterilization of grain before germination with sodium hypochlorite (Table). The number of bacteria not sensitive to the various antibiotics or their combinations were in such cases always less than 10^3. Worth attention is the fact that the presented effectiveness of sterile preparation of wheat seedlings after treatment of the grain with hypochlorite was obtained with germination of the grain on perlit. If the germinations was, however, in water the bacteria on the seedlings were approximately as numerous as on control seedlings grown from not sterilized grain. Similar results were also obtained by Lonberg-Holm (1967) for the germination in water of lettuce seeds sterilized in advance with hypochlorite. These results show that germination in water creates favourable conditions for the intensive development of bacterial flora from the bacteria remaining on the material after sterilization.

The presented results allow the following conclusions:

1. On wheat seedlings grown from grain not sterilized in advance occur large numbers of bacteria exceeding 10^8 microorganisms per one gram fresh weight. It thus seems necessary in studies on the incorporation of radioactive precursors into the nucleic acids of grain seedlings to employ treatment eliminating the influence of the bacteria on the results.

Table 1

Action of streptomycin, penicillin G*, chloramphenicol and various combinations of these antibiotics against the bacteria on wheat seedlings grown from grain sterilized with hypochlorite and not sterilized grain: either treated with detergents or not. For further explanations see text

No.	Antibiotic content in medium mg/l	Number of colony forming bacteria, in millions per 1 gram fresh weight			
		grain before germination:			
		not sterilized			sterilized with sodium hypochlorite
		not washed seedlings	washed seedlings		
			0,1 % CTA--Br**	2% SLS***	
1.	—	488	23	38	below 0.01
2.	Streptomycin 50	355	10	7.1	,, 0.001
3.	,, 100	236	7.1	4.8	,, ,,
4.	,, 200	278	4.8	4.6	,, ,,
5.	Chloramphenicol 50	46	2.9	5.3	,, ,,
6.	,, 100	28	0.8	3.0	,, ,,
7.	,, 200	8.1	0.4	1.8	,, ,,
8.	Penicillin G 50	284	15	25	,, ,,
9.	,, 100	160	15	26	,, ,,
10.	,, 200	72	3.9	7.6	,, ,,
11.	Streptomycin 50 Chloramphenicol 50	4.2	0.1	0.2	,, ,,
12.	Streptomycin 50 Penicillin G 50	127	4.9	4.0	,, ,,
13.	Chloramphenicol 50 Penicillin G 50	30	2.1	3.6	,, ,,
14.	Streptomycin 50 Chloramphenicol 50 Penicillin G 50	1.4	0.1	0.2	,, ,,
15.	Streptomycin 20 Chloramphenicol 25 Penicillin G 25	37	0.6	0.5	,, ,,
16.	Streptomycin 100 Chloramphenicol 100	0.3	0.01	0.07	,, ,,

The results presented in the table are mean values from 5 experiments.
* — potassium salt of penicillin G, 1500 in units/mg,
** — cetyltrimethylammonium bromide,
*** — sodium lauryl-sulphate

2. Of the antibiotics (penicillin, chloramphenicol, streptomycin) the most effective against the bacteria on wheat seedlings is chloramphenicol coupled with streptomycin and when employed singly — chloramphenicol. The one of these antibiotics against the bacteria on wheat seedlings grown from grain not sterilized in advance does not ensure proper conditions of sterility for the above mentioned studies.

3. The treatment of wheat seedlings with such detergents as cetyltrimethylammonium bromide and sodium lauryl sulphate results in conside-

rable, over ten-fold reduction in the number of bacteria. The use of these agents, due to the absence of data on their possible action against plant material, should be preceded by additional studies.

4. Sterilization of grain with sodium hypochlorite prior to planting results in seedlings with high degree of sterility in the conditions of germination on solid substratum (sterile perlit), as opposed to germination in water.

5. Worth recommendation is control of both the sterility of plant material prior to each experiment in studies on the incorporation of radioactive precursors into the plant's nucleic acid and — as in the case of antibiotics — the effectiveness of their action against bacteria.

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Skuteczność różnych sposobów sterylnego przygotowywania siewek zbóż do badań kwasów nukleinowych z zastosowaniem radioaktywnych prekursorów

Streszczenie

Ziarniaki pszenicy nie sterylizowane oraz sterylizowane podchlorynem sodu wysiewano w sterylnych warunkach na szalki Petriego lub do perlitu. Kiełkowanie przeprowadzano w ciemności przez 4 doby. Siewki wyrosłe z ziarniaków nei steryliowanych przemywano przez 15 minut 0,1% roztworem bromku cetylotrójmetylo-amoniowego lub 2% siarczanem laurylo-sodowym. Stopień zanieczyszczenia siewek bakteriami określano techniką posiewów na podłożach agarowych na szalkach Petriego. Skuteczność działania chloramfenikolu, streptomycyny i penicyliny w różnych stężeniach (50—200 mg/1 l) oraz ich kombinacji przeciwko bakteriom siewek pszenicy o różnym stopniu sterylności badano przez dodawanie antybiotyków do podłoży agarowych.

Stwierdzono występowanie na siewkach pszenicy, wyrosłych z ziarniaków nie sterylizowanych, bakterii w ilościach powyżej 10^8 sztuk/1 g. św. m. Stosowanie bromku cetylotrójmetylo-amoniowego lub siarczanu laurylo-sodowego powodowało kilkunastokrotne zmniejszenie ilości bakterii występujących na siewkach.

Siewki o wysokim stopniu sterylności (poniżej 10^4 szt. bakterii/1 g. św. masy) uzyskiwano w przypadku przedsiewnej sterylizacji ziarniaków podchlorynem i przeprowadzania kiełkowania w podłożu stałym (perlit). Natomiast w wyniku kiełkowania w wodzie uzyskiwano siewki o zawartościach bakterii, zbliżonych do ich ilości, występujących na siewkach kontrolnych, wyrosłych z ziarniaków nie sterylizowanych. Spośród stosowanych antybiotyków najbardziej skuteczne działanie przeciwko bakteriom siewek pszenicy wykazywał chloromfenikol w kombinacji ze streptomycyną, a z antybiotyków stosowanych oddzielnie — chloramfenikol.

Zaleca się zarówno każdorazowe sprawdzania sterylności materiału roślinnego podczas badań nad włączaniem radioaktywnych prekursorów do jego kwasów nukleinowych, jak i — w przypadku stosowania antybiotyków — skuteczności ich oddziaływania na bakterie zanieczyszczające dany materiał.