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BIOASSAY FOR SELECTION OF BIOCONTROLLER BACTERIA AGAINST BEAN COMMON BLIGHT (*XANTHOMONAS AXONOPODIS* PV. *PHASEOLI*)

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ABSTRACT

Emphasis has been given on selection of micro-organism for biological control. However, in order to evaluate the biological control potential of a great number of micro-organisms in a small period of time it is necessary to develop an efficient bioassay. Seven hundred and sixty bacterial isolates from different habitats, were selected for compatibility with *Rhizobium leguminosarum* bv. *phaseoli* (SEMIA 4077 e SEMIA 4080). Among them 596 isolates were ineffective against both rhizobia. Bean seeds immersed in suspension of each one of these isolates were agitated for 5 hours at 10ºC and sowed in non-sterilized soil. The plants were kept in greenhouse. After the development of cotyledonary and primary leaves, these were removed and bioassayed for *Xanthomonas axonopodis* pv. *phaseoli* (XAP) control. In the cotyledonary leaves, it was observed that the isolate DFs093 offered 100% control, DFs041 and DFs1297 offered 90% and DFs490, DFs769, DFs831, DFs842 and DFs843 offered 80% control. In the primary leaves, the DFs482 isolated offered 100% and the DFs080, DFs348, DFs513, DFs622, DFs769, DFs842 and DFs912 offered 80% of XAP control.

Key words: Biological control, antagonism, *Phaseolus vulgaris*, microbiolization

INTRODUCTION

Bean (*Phaseolus vulgaris* L.) is one of the most cultivated legume, representing one of the main sources of nutrients in the diet of the Brazilian population (3). It is a crop very adaptable in terms of edaphyc and climatic conditions, taking part in the productive systems of small and medium farmers, whose production is directed to family consumption and to commercing the excess (29).

According to Paul and Clark (19) bean has the capacity to form symbiotic association with the bacterium *Rhizobium leguminosarum* biovar *phaseoli*. The population of rhizobium in the soil interacts with a series of biological factors: nutrients competition, predation, parasitism and antibiosis that could considerably limit the rhizobium population in the soil (13). Therefore the antagonist microrganisms used as agents of biological control must have, a neutral or synergic relationship with the benefic bacteria that live in the plants roots, especially, the rhizobium.

The bacterial bean common blight (BCB) caused by *Xanthomonas axonopodis* pv. *phaseoli* is a disease which causes a low yield. The BCB is a hard to control disease. The chemical control, by spraying the plants in the field, has demonstrated no efficiency (15) and is economically impracticable. Therefore the recommended control is the use of seeds of good sanitary quality (5), use of resistant cultivars (27) and cultural practices as crop rotation, weed elimination and incorporation or elimination of debris (24). These procedures could be complemented by the utilization of antagonist microrganisms in seed or vegetative part treatment, that represented a suitable alternative with low health risks for farmers, consumers and environment.

A biological control program is based on the selection of antagonistic microorganisms *in vi*tro or *in vivo*, by bioassay at laboratory conditions or in greenhouse in the presence of both phytopathogen and host plant, entire or detached organs (14).

Based on latter strategies, the aim of this work was to develop a bioassay to select compatible bacteria with *Rhizobium* of bean,

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and at the same time, capable of controlling *X. axonopodis* pv. *phaseoli*.

MATERIAL AND METHODS

Media: Actinomycetes were isolated in soil extract medium (21): glucose (1 g), K_2HPO_4 (0,5 g), KNO_3 (0,1 g) and soil extract (100 mL), agar (20 g) for 900 mL of distilled water, added with cycloheximide at 100 mg.L^{-1} final concentration (28) and phenol at 0,7 mg.L^{-1} (11). Other soil bacteria were isolated in 523 medium (10): sucrose (10 g), casein (8 g), yeast extract (4 g), K_2HPO_4 (2 g), $MgSO_4.7H_2O$ (0,75 g), agar (20 g), distilled water (1000 mL), modified by adding cycloheximide into the concentration of 100 mg.L^{-1}.

Bacteria isolation: Suspensions were prepared with 10 g of: soil or leaves or flowers, or pods, or seeds of different bean cultivars from different areas, added to 90 mL of saline solution (0,85% NaCl), under agitation for 30 minutes. Samples suspensions were submitted to serial dilutions, of 100 µL each (10^{-3}, 10^{-4} and 10^{-5}) and inoculated in Petri dishes. Petri dishes were incubated at 28ºC up to 21 days, being daily observed. Colonies with different morphological characteristics were transferred to tubes containing soil extract media (actinomycetes) or 523 media (other bacteria).

***Rhizobium* strains:** The *R. leguminosarum* bv. *phaseoli* strains used were SEMIA 4077 (isolate 0300, FEPAGRO) and SEMIA 4080 (isolate 9/98 FEPAGRO) from Microbial Resources Centers (MIRCENS) [Agronomic Research Fundation of Rio Grande do Sul (FEPAGRO) - Nitrogen Fixation Center, Porto Alegre, RS].

Pathogenic isolate: The isolate of *X. axonopodis* pv. *phaseoli* XAP 28 was provided by Temperate Weather Research Center (Embrapa-CPACT), Pelotas, RS. The pathogenicity of the isolate was evaluated using the leaf sectioning inoculation method with scissors, as described by Dhingra and Sinclair (7).

"In vitro" compatibility between isolated bacteria and *R. leguminosarum* bv. *phaseoli* strains: Petri dishes were added of agar water 2%, just enough to fill the bottom. With a leveled layer, 10 mL of molten medium 523 containing 1% of cell suspension of the rhizobium was added. After solidification, six sterilized filter disks previously dived into 72 h old liquid culture of each of the 760 bacteria to be tested were added, being equidistantly deposited. The plates were incubated for 72 h at 28ºC. After this period, the plates were checked for the presence or absense of rhizobium growing inhibition halo, indicating the occurrence of antibiosis between the microrganisms tested.

Detached leaves bioassay selection of isolates with *X. axonopodis* pv. *phaseoli* biocontrol potential: To evaluate biocontrol potential, 596 isolates, from different habitats, all compatible with *R. leguminosarum* bv. *phaseoli* were used. Bean seeds from the Engopa Ouro cultivar were individually immersed in saline solution of each isolate. The bacteria were previously cultivated in the 523 medium for 48 h, and the suspension concentration adjusted to A_{540}= 0.50. The seeds were agitated during 5 h at 10ºC. Seeds immersed on saline solution were used as a control. Seven seeds were sowed in pots containing 1 Kg of non-sterilized soil, bovine manure and sand in the 3:1:1 proportion. Plants were maintained in a greenhouse with 4 plants for each pot. After development, cotyledonary and primary leaves were removed, identified and inoculated, being deposited in gerboxes, previously disinfested with alcohol 70%, hypochlorite 2,5% and washed with distilled water, containing two wet paper sheets. Ten (10) scissors cuts were made in these leave's boards, followed by the deposition of pathogen suspension (10 µL). The incubation was made at 24ºC for 10 days under fluorescent lamps illumination for 12 h/day. The evaluation was made 10 days after pathogen inoculation by visual observation of the BCB symptoms of BCB. Test of exudation in drops (12) were made for confirmation of bacterial etiology. The control percentage was calculated considering the amount of cuts that didn't developed infection.

Isolates preservation: All isolates were preserved by consecutive transferences in 523 medium, being stored at 4ºC. The compatible cultures with both rhizobium tested were also preserved in sterile soil, distilled water and emulsified in glycerin (7).

RESULTS

A total of 332 antagonists from different bean cultivars and/or different locations were isolated: 76 from soil, 224 from the aerial part and 32 from seeds. Other 428 isolates from different habitats were obtained in the collection of the Bacteriology Laboratory (Phytossanity Department – Pelotas Federal University) making a total of 760 antagonists.

Concerning the compatibility with *R. leguminosarum* bv. *phaseoli* (SEMIA 4077 and SEMIA 4080), 596 isolates, corresponding to 78.4% of the total evaluated, did not form any inhibition halo (data not shown), indicating no activity against the lineages of rhizobium.

Antibiotic production was observed in 164 isolates from different habitats, which were able to inhibit the growth of one or both lineages of rhizobium, forming clear inhibition halos. The SEMIA 4080 lineage showed higher sensitivity to the substances produced by the evaluated isolates, being inhibited by 113 out of 760 isolates (15%). Inhibition of only SEMIA 4080 was observed in 82 isolates and a small number of isolates was capable of inhibiting the growth of both lineages, corresponding to 4,0% of the total of tested.

Among 596 isolates, from different habitats and compatible with rhizobium, 226 did not allow seed germination or plantlets emergency, even after four attempts. Therefore, these isolates could not be evaluated for biological control potential. The 370

remaining isolates were evaluated for cotyledonary and primary leaves, except 44 isolates that didn't permit the development of primary leaves.

A few isolates offered high percentage of biological control (Table 1) and only eigth isolates (2.16% of the total evaluated in the bioassay and 1.05% of the total of isolates evaluated for *R. leguminosarum* bv. *phaseoli* compatibility) presented more than 80% of control.

In the cotyledonary leaves evaluation (Table 2), it was observed that DFs093 soil isolate, offered 100% of control, DFs041 (garlic tunic) and DFs1297 (selected for *Ralstonia solanacearum* control in tomato plant), offered 90%, and the isolates DFs490 (endophytic of garlic), DFs769 (bean pod), DFs831 (soil planted with beans), DFs842 and DFs843 (bean leaf) offered 80% of control for *X. axonopodis* pv. *phaseoli*.

In the primary leaves (Table 2) it was possible to observe that the DFs482 isolate (garlic tunic) offered 100% and the DFs080 isolate (soil cultivated with garlic), DFs348 (onion phylloplane), DFs513 (tunic of onion), DFs622 (soil), DF769 (bean pod), DFs842 and DFs912 (bean leaf) offered 80% of *X. axonopodis* pv. *phaseoli* control (Table 2).

Table 1. Control of *X. axonopodis* pv. *phaseoli* inoculated by foliar sectioning in detached leaves from plants originated when seeds were microbiolizeted with isolates compatible with *R. leguminosarum* bv. *phaseoli*.

Porcentage of control	Number of isolates	
	Cotyledonary leaves	Primary leaves
0	110	105
10	47	37
20	70	55
30	42	32
40	33	38
50	32	21
60	14	15
70	13	15
80	5	7
90	2	0
100	1	1

Table 2. Identity of isolates from different habitats with potential for *X. axonopodis* pv. *phaseoli* biological control.

Code	Identification	Habitat	control % in cotiledonary leaves	control % in primary leaves
DFs41		Tunic of garlic	90	0
DFs80	*Pseudomonas* sp.	Garlic rhizosphere	30	80
DFs93	*Bacillus cereus*	Soil	100	0
DFs348	*Bacillus*	Onion phylloplane	0	90
DFs482		Tunic of garlic	20	100
DFs490		Garlic endophytes	80	0
DFs513	*Pseudomonas*	Tunic of onion	40	80
DFs622	*Bacillus*	Bean rhizosphere	0	80
DFs769	*Bacillus cereus*	Bean pod	80	80
DFs831	*Pseudomonas*	Bean rhizosphere	80	60
DFs842	*Pseudomonas*	Bean phylloplane	80	80
DFs843	*Rhodococcus fascians*	Bean phylloplane	80	30
DFs912	*Rhodococcus fascians*	Bean phylloplane	70	100
DFs1297		Soil	90	30

DISCUSSION

Patel (18) evaluated 279 actinomycetes against 12 lineages of rhizobium, using agar discs, and failed to detect antagonistic activity. In soybean, problems related to nodulation were attributed to the predominance of antibiotic producing actinomycetes (25). Tests to evaluate the antimicrobial spectrum (20) indicated that the actinomycetes isolates influenced the capacity of lineages of *Bradyrhizobium* spp to compete for soybean nodulation sites. On the other hand, rhizobium in seeds of beans co-inoculated with *Bacillus subtilis* presenting biological control capacity against soil fungi did not interfere in the growth promotion capacity (8), showing that the development of mixed inoculants could be useful to promote growth (rhizobium effect by nitrogen contribution) and to protect plants from diseases (*Bacillus* effect by inhibition of pathogenic fungi).

The bioassay using bean detached leaves under controlled conditions allowed to evaluate a wide range of isolates, maintaining the leaf color and the turgesce of the tissues. In many cases, there was a development of callus and root system, and the occurrence and intensity of symptoms was easily verifiable (Fig. 1). Other positive aspects were the use of known and standardized inoculum quantity during the whole evaluation and the possibility of evaluating each bean leave separately. There are several papers on bioassays for selection of microrganisms with potential biological control, including potato slices for selection of antagonists against *Erwinia carotovora* subsp. *atroseptica* (22), detached bean leaves for

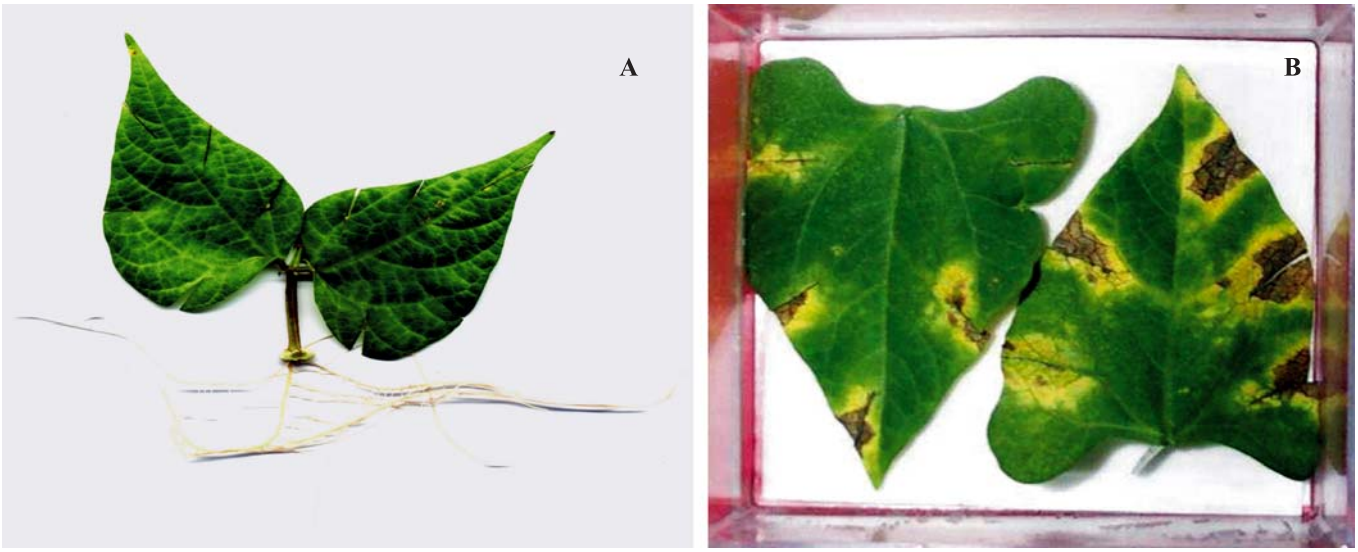

Figure 1. Detached bean leaf, after 10 days of incubation, showing callus development and root system (A) and leaves inoculated with *Xanthomonas axonopodis* pv. *phaseoli* with typical bean common blight symptoms (B).

biological control of beans rust caused by *Uromyces phaseoli* (1, 2, 4, 16) and geranium rust (23), leaf discs of garden rose for control of *Botrytis cinerea* (26), and *Kalanchoe tubiflora* leaves for control of *Agrobacterium tumefascens* (17).

Among the bacteria (Table 2), there are some isolates of the genera *Bacillus* and *Pseudomonas*, that are good biocontrollers, including some used as commercial products (9,30). The safety of the use of microorganisms for biocontrol was discussed by Cook *et al.* (6), who showed that despite the existing risks, *Bacillus* and *Pseudomonas* do not present pathogenicity, allergenicity or toxigenic effect on people, domestic animals and wildlife. Besides this, the populations of microorganisms applied to the environment commoly often decline to undetectable levels (6).

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RESUMO

Bioensaio para seleção de bactérias biocontroladoras do crestamento bacteriano comum do feijão (*Xanthomonas axonopodis* pv. *phaseoli*)

Tem-se dado muita ênfase ao controle biológico mediante seleção de microorganismos. Porém, para se avaliar o potencial de biocontroladores de forma massal e em pequeno intervalo de tempo é necessário desenvolver um bioensaio eficiente. Bactérias de diferentes sítios, num total de 760 isolados, foram selecionadas para compatibilidade com *Rhizobium leguminosarum* bv. *phaseoli* estirpes SEMIA 4077 e SEMIA 4080, onde 596 isolados foram inefetivos contra ambos rizóbios. Sementes de feijão foram imersas em suspensão de cada um destes isolados sendo agitadas por 5 horas a 10ºC, plantadas em solo não esterelizado, sendo as plantas mantidas em casa de vegetação. Após o desenvolvimento das folhas cotiledonares e folhas primárias, estas foram retiradas e avaliadas por bioensaio para o controle de *Xanthomonas axonopodis* pv. *phaseoli* (XAP). Nas folhas cotiledonares, observou-se que o isolado DFs093, proporcionou 100% de controle, DFs041 e DFs1297 propiciaram, 90% e DFs490, DFs769, DFs831, DFs842 e DFs843 proporcionaram 80% de controle. Nas folhas primárias, o isolado, DFs482 propiciou 100% e os isolados DFs080, DFs348, DFs513, DFs622, DFs769, DFs842 e DFs912 proporcionaram 80% de controle para XAP.

Palavras-chave: Controle biológico, antagonistas, *Phaseolus vulgaris*, microbiolização

REFERENCES

1. Baker, C.J.; Stavely, J.R.; Thomas, C.A.; Sasser, M.; Macfall, J.S. (1983). Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and development of rust pustules on bean leaves. *Phytopath.*, 73(8), 1148-1152.
2. Bettiol, W.; Varzea, V.M.P. (1992). Biological control of coffee leaf rust (*Hemileia vastatrix*) by *Bacillus subtilis* under controlled conditions. *Fitopatol. Bras.*, 17(1), 91-95.

3. Cavalcanti, L.S.; Coelho, R.S.B.; Perez, J.O. (2002). Utilização de dois métodos de inoculação na avaliação da resistência de cultivares e linhagens de feijoeiro a *Fusarium oxysporum* pv. *phaseoli*. *Cienc. Rural.*, 32(1), 1-5.
4. Centurion, M.A.P.C.; Kimati, H. (1994). Biological control of the bean rust with antagonistic bacteria. *Summa Phytopathol.*, 20(3-4), 179-183.
5. Coppeland, L.O.; Adams, M.W.; Bell, D.C. (1975). A improved seed programme for maintaining disease-free seed of field beans (*Phaseolus vulgaris*). *Seed Sci. Tech.*, 3, 719-724.
6. Cook, R.J.; Bruckart, W.L.; Coulson, J.R.; Goettel, M.S.; Humber, R.A.; Lumsden, R.D.; Maddox, J.V.; McManus, M.L.; Moore, L.; Meyer, S.F.; Quimby JR., P.C.; Stack, J.P.; Vaughn, J.L. (1996). Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biol. Control.*, 7, 333-352.
7. Dhingra, O.D.; Sinclair, J.B. (1995). *Basic plant pathology methods*. 2nd ed. CRC Press, Boca Raton, 434p.
8. Jensen, C.E.; Percoch, J.A.; Graham, P.H. (2002). Integrated management strategies of bean root rot with *Bacillus subtilis* and *Rhizobium* in Minnesota. *Field Crops Res.*, 74, 107-115.
9. Jacobsen, B.J.; Zidak, N.K.; Larson, B.J. (2004). The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. *Phytopath*, 94, 1272-1275.
10. Kado, C.I.; Heskett, M.G. (1970). Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *Xanthomonas*. *Phytopath.*, 60, 24-30.
11. Lawrence, C.H. (1956). A method of isolation of actinomycetes from scaby potato tissue and soil with minimal contamination. *Can. J. Bot*., 34, 44-47.
12. Lelliot, R.A.; Stead, D.E. (1987). *Methods of diagnosis of bacterial diseases of plants.* Blackwell Scientific Publications, Oxford, 216p.
13. Lovato, P.E.; Pereira, J.C.; Vidor, C. (1985). Flutuação populacional de estirpes de *Rhizobium phaseoli* na rizosfera de feijão. *Rev. Bras. Ciênc. Solo*, 9, 211-218.
14. Mariano, R.L.R. (1993). Métodos de seleção *in vitro* para controle microbiológico. *Revisão Anual de Patologia de Plantas*, 1, 369-409.
15. Maringoni, A.C. (1990). Controle químico do crestamento bacteriano comum do feijoeiro e seu efeito na transmissão de *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye pelas sementes. *Pesq. Agropec. Bras.*, 25(8), 1151-1156.
16. Mizubuti, E.S.G.; Maffia, L.A.; Muchovej, J.J.; Romeiro, R.S.; Batista, U.G. (1995). Selection of isolates of *Bacillus subtilis* with potential for the control of dry bean rust. *Fitopatol. Bras.*, 20(4), 540-544.
17. Napoleão, R.; Romeiro, R.S.; Berian, L.O.S.; Barbosa, J.G. (1998). A bioassay for rapid screening of bacteria antagonistic to *Agrobacterium tumefaciens*. *Fitopatol Bras.*, 23(1), 27-29.
18. Patel, J.J. (1974). Antagonism of actnomycetes against rhizobia. *Plant Soil.*, 41, 395-402.
19. Paul, E.A.; Clark, F.E. (1989). *Soil microbiology and biochemistry*. Academic Press, San Diego, 273 p.
20. Pereira, J.C.; Neves, M.C.P.; Drozdowicz, A. (1999). Influência da antibiose exercida por actinomicetos às estirpes de *Bradyrhizobium* spp., na nodulação da soja. *Pesq. Agropec. Bras*., 34(1), 99-108.
21. Pramer, D.; Schmidt, E.L. (1964). *Experimental soil microbiology*. Burgess Publishing Company, Minnesota.
22. Rhodes, D.; Logan, C.; Gross, D.C. (1986). Selection of *Pseudomonas* spp. inhibitory to potato seed tuber decay. *Phytopath.*, 76(10), 1078.
23. Rytter, J.L.; Lukezic, R.L.; Craig, R.; Moorman, G.W. (1989). Biological control of geranium rust by *Bacillus subtilis*. *Phytopath.*, 79, 367-370.
24. Saettler, A.W. (1991). Common bacterial blight. *In*: Hall, R. (ed.). 2nd ed. *Compendium of bean diseases*. APS Press, St. Paul, p.29-30.
25. Scotti, M.M.M.L.; Sá, N.M.H.; Vargas, M.A.T.; Dobereiner, J. (1988). Avaliação da resistência a tetraciclina, kanamicina, cloranfenicol de estirpes de *Bradyrhizobium japonicum* e *Bradyrhizobium* de *Stylosanthes* isoladas de solo de cerrados. *Rev. Microbiol.*, 19(1), 60-66.
26. Tatagiba, J.S.; Maffia, L.A.; Barreto, R.W.; Alfenas, A.C.; Sutton, J.C. (1998). Biological control of *Botrytis cineres* in residues and flowers of rose (*Rosa hybrida*) *Phytoparasitica*, 26(1), 8-19.
27. Webster, D.M.; Temple, S.R.; Schwartz, H.F. (1980). Selection for resistance to *Xanthomonas phaseoli* in dry beans. *Crop Sci.*, 20, 519-522.
28. Willians, S.T.; Davies, F.L. (1965). Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. *J. Gen. Microbiol.*, 38, 251-261.
29. Yokoyama, L.P.; Banno, K.; Kluthcouski, J. (1996). Aspectos socio-econômicos da cultura *In:* Araujo, R.S. (ed.) *Cultura do feijoeiro comum no Brasil*. *Potafos*, Piracicaba, p.1-21.
30. Wash, U.F.; Morrissey, J.P.; O'Gara. (2001). F. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial explotation. *Environ. Biotech.*, 12, 289-295.