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Short communication

Factors affecting callus and shoot formation from in vitro cultures of *Lens culinaris* Medik.

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Abstract. The influence of culture medium and explant on callus and shoot formation of lentil (*Lens culinaris* Medik.) has been studied. Three different explants (shoot-tip, first node and first pair of leaves) from three Spanish lentil cultivars were cultivated on two basal media: Murashige and Skoog medium (MS) and medium with mineral salts of MS medium plus vitamins of Gamborg's B5 medium (MSB), supplemented with growth regulators. Media with 2,4-D induced the formation of calli in all explants, but no organ regeneration was obtained from these calli. Multiple shoot formation was obtained from 33% to 92% of the explants in media supplemented with 2.25 $mg\,l^{-1}$ of BA and 0.186 $mg\,l^{-1}$ NAA + 2.25 $mg\,l^{-1}$ BA; in the other media one to two shoots per explant were formed in 10 to 98% of the explants. Root formation from explants was achieved only in media with NAA or IAA. Of the explants tested, the best morphogenetic responses were obtained from nodes and the poorest from leaves.

Introduction

In recent years the interest in tissue culture research of legumes has increased considerably. Plant regeneration from somatic cell in vitro culture has been difficult to achieve among large seeded leguminous species, in contrast to forage legumes [1]. Successful plant regeneration has been reported in several species of grain legumes such as *Arachis hypogaea*, *Phaseolus vulgaris*, *Cicer arietinum*, *Vigna unguiculata*, *Glycine max*, *Glycine soja*, *Vicia faba* and *Pisum sativum* [2–8]. Knowledge of the optimal conditions which favour differentiation under in vitro conditions is an important step in the application of in vitro manipulation techniques in higher plants. Successful plant regeneration depends on factors such as the genotype, the explant and the composition of medium, especially growth regulators.

Lentil (*Lens culinaris* Medik.) is a grain legume of great economic value,

and hence of research interest. It has been cultivated in semi-arid areas of the Mediterranean Basin and Asia throughout the ages, and its contribution to human nourishment is of vital importance in some areas. However, it has been one of the least used legumes in studies of in vitro cultures. Bajaj & Dhanju [9] first reported in vitro lentil regeneration from meristem tips. Williams & McHugen [10] regenerated shoots from callus after culturing explants of the lentil cultivar Eston in MS media with kinetin and giberellic acid. Finally, Saxena & King [11] regenerated lentil plants from callus cultures of the cultivar Laird via somatic embryogenesis. The present paper reports new data on the effect of explant and media composition and callus initiation and morphogenetic responses in three Spanish cultivars of lentil.

Materials and methods

Seeds of three Spanish cultivars of lentil (*Lens culinaris* Medik.), Verdina, Pardina and Castellana, were used for this study. Seed samples were provided by the Plant Breeding Station of Valladolid (SIA), Spain. The seeds were surface-sterilized by immersion (2 s) in 70% ethanol and then in 5% sodium hypochlorite solution (20 min). After two rinses in sterile distilled water, the seeds were inoculated in sterile flasks containing wet cotton wool. After 8 days, shoot-tip (4–7 mm), first node and the first pair of leaves (hereafter they will be referred to as shoot-tip, node and leaves, respectively) were removed from seedlings. The explants were inoculated onto different media. Two basal media were used:

- Murashige & Skoog's [12] medium (MS), pH 5.6, Difco Bacto-agar ($10\,g\,l^{-1}$), sucrose ($30\,g\,l^{-1}$), and
- MSB medium which consisted of the mineral salts of MS medium plus the vitamins of the Gamborg's B5 medium [13], pH 5.8, Bacto-agar ($8\,g\,l^{-1}$), sucrose ($30\,g\,l^{-1}$).

Both media were supplemented with the growth regulators showed in Table 1. The number of explants used and concentrations of growth regulators are also shown in Table 1. The media were autoclaved at 15 psi, 121 °C for 17 min. All the cultures were incubated at 25 ± 1 °C under a light regime of 16 h (fluorescent tubes Sylvania F36W/GRO, light intensity $2.94\,W\,m^{-2}$).

The explants were cultured for four weeks. Shoots were then isolated and were transferred to flasks containing MS basal medium with NAA ($0.0186\,mg\,l^{-1}$), BA ($0.225\,mg\,l^{-1}$) and GA_3 ($1\,mg\,l^{-1}$) to promote further shoot growth. Residual explants and/or calli were transferred to fresh medium of the same composition.

Results and discussion

Callus formation

Irrespective of the growth regulator combination all the media induced callus formation. Table 1 shows the responses obtained in the different culture media after four weeks. The media with 2,4-D gave 100% callus formation irrespective of the cultivar and explant, and the greatest cullus growth, but were ineffective in inducing morphogenesis. Similar results were obtained by Mok & Mok [14] and Ruiz et al. [15] in *Phaseolus*, and Rubluo et al. [4] in *Pisum*. All media without 2,4-D induced callus formation, but at lower frequency than in media with 2,4-D. This callus grew relatively slowly. Media containing IAA generally produced the least callus formation and growth. Likewise, media containing BA as the only phytohormone induced callus formation at a low frequency, while, on the contrary, media with NAA + BA gave callus at a high frequency. In general no noticeable differences in callus formation and growth were observed between the two basal media, MS and MSB, except for the growth regulator combination of NAA + BA where MSB was superior.

Verdina generally formed callus at a lower frequency than the two other cultivars in media without 2,4-D, while the percentages between Castellana and Pardina varied from medium to medium. The influence of the explant was less pronounced, although the node generally formed calli with the highest frequency and the leaf with the lowest.

Morphogenesis

2,4-D, alone or in combination with any other auxin and/or cytokinin, did not induce morphogenesis (Table 1). In contrast, regeneration of plants has been successfully achieved using 2,4-D in other leguminous species such as *Trifolium pratense* [16] and *Arachis hypogaea* [17], to mention but a few. Nevertheless our data agree with those of Williams & McHugen [10] with *Lens culinaris*.

Of the growth regulators tested, BA was the most effective inducing shoot formation from calli or directly from the explants (shoot-tip and node). This was due not so much to the frequency of regeneration, which was not always the highest, but to the kind of morphogenesis obtained in the form of multiple shoots (Fig. 1). Combinations involving NAA and BA have also been successfully employed in inducing plant regeneration in other legumes [2, 3, 5, 7, 18]. The shoot-tips and nodes inoculated onto the media with NAA or IAA resulted in the formation of one or two shoots per explant,

Table 1. In vitro response of lentil explants cultured in different media. The data are the frequency of explants giving a particular response from cultures scored after four weeks.

Basal media	Hormones ($mg\,l^{-1}$)	Number of explants, and cultivar[1]	Response (%)								
			Callus			Shoot			Root		
			S[2]	L	N	S	L	N	S	L	N
MS	2,4-D(2)	50 C	100	100	100	0	0	0	0	0	0
		54 V	100	100	100	0	0	0	0	0	0
		48 P	100	100	100	0	0	0	0	0	0
	BA(2.25)	74 C	18	8	18	74	0	91	0	0	0
		70 V	0	0	0	84	0	90	0	0	0
		61 P	16	0	16	66	0	89	0	0	0
	IAA(2)	46 C	0	7	0	27	0	77	72	8	54
		44 V	2	0	0	54	0	57	86	0	25
		40 P	22	34	38	43	0	70	68	4	15
	NAA(0.186)	49 C	10	20	16	10	0	84	80	37	82
		52 V	2	5	13	27	0	67	86	12	48
		56 P	5	38	41	20	0	36	64	7	27
	NAA(0.186) +	55 C	34	45	65	60	0	90	0	0	0
	BA(2.25)	85 V	6	19	46	81	0	91	0	0	0
		64 P	38	34	47	53	0	75	0	0	0
	2,4-D(2) +	53 C	100	100	100	0	0	0	0	0	0
	BA(0.9)	44 V	100	100	100	0	0	0	0	0	0
		48 P	100	100	100	0	0	0	0	0	0
	2,4-D(2) +	51 C	100	100	100	0	0	0	0	0	0
	BA(0.8) +	59 V	100	100	100	0	0	0	0	0	0
	IAA(2)	52 P	100	100	100	0	0	0	0	0	0

MSB	BA(2.25)	54 C	31	6	37	52	0	41	0	0	2
		50 V	2	0	2	33	0	60	0	0	0
		40 P	8	0	5	40	0	50	0	0	0
	IAA(2)	48 C	6	18	40	40	0	98	54	10	19
		30 V	0	0	0	17	0	67	32	22	98
		30 P	0	19	0	13	0	64	50	27	73
	NAA(0.186)	54 C	16	17	22	12	0	47	35	17	30
		30 V	7	7	0	13	0	82	17	0	43
		30 P	8	4	20	20	0	60	29	0	27
	NAA(0.186) +	51 C	69	90	82	55	0	92	0	0	0
	BA(2.25)	48 V	95	98	100	61	0	67	0	0	0
		46 P	93	98	93	59	3	67	0	0	0

[1] C = Castellana; V = Verdina; P = Pardina.
[2] S = shoot-tip; L = leaf; N = node.

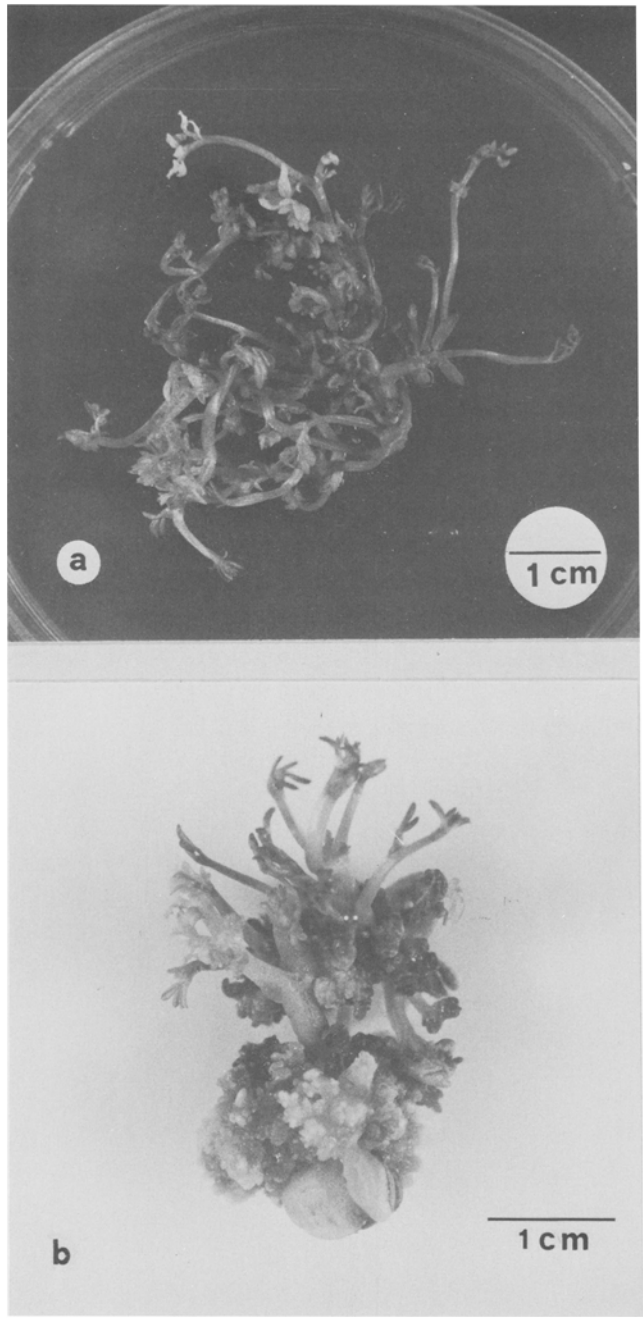

Fig. 1. Multiple shoot formation from Verdina node. (*a*) in MS + BA medium, (*b*) in MSB + NAA + BA medium.

which may have originated from the pre-existing meristems in the original explants [17].

Our results agree with the reported inhibitory effect of BA on the formation of roots [2]. The media used in our study containing BA alone or in combination with NAA never induced roots. In the media with IAA alone, roots were large and frequent, while in media with NAA they were short, thick, and resembling calli.

Leaves gave the poorest response since no shoots were obtained irrespective of the culture media and cultivar tested, except a single explant of Pardina on MSB + BA + NAA medium. Nodes gave the best response. Williams & McHugen [10] also observed that in lentil the ability to regenerate shoots varied from one explant to another. They obtained the best regeneration response (60%) culturing shoot meristems in a MS + kinetin + gibberellic acid medium, while we have obtained an 84% morphogenetic response from shoot-tips and a 98% from nodes, but in media different from the ones used by Williams & McHugen [10]. Saxena & King [11] obtained lower frequencies (3–5%) of plant regeneration via somatic embryogenesis from embryo-derived callus of lentil.

The formation of roots occurred less frequently from leaf explants than from shoot-tips or nodes. The frequency of root formation from shoot-tips and nodes were similar, but their frequency of root formation depended on the cultivar and medium used (Table 1).

The shoots regenerated were transferred to MS basal medium with NAA + BA + GA_3 to promote shoot elongation. In this medium the shoots grew but we have not observed rooting. We have also failed to induce rooting from regenerated shoots in MS basal medium (i.e. growth-regulator-free) and MS medium supplemented with 2 mg l^{-1} of IAA or 0.186 mg l^{-1} of NAA. Thirty regenerated shoots were transferred to each of these last three media.

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