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CALLUS INDUCTION AND PLANTLET REGENERATION FROM *IN VIVO* NODAL AND INTERNODAL SEGMENTS AND SHOOT TIP OF *DALBERGIA SISSOO* ROXB.

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Abstract

A complete and efficient protocol was developed for *in vitro* callus induction, somatic embryogenesis and plantlet regeneration from *in vivo* nodal and internodal segments and shoot tips of sissoo (*Dalbergia sissoo* Roxb.). The highest percentage of callus induction (100%) was achieved from nodal segment on MS medium supplemented with 2.0 mg/1 BAP + 0.5 mg/1 NAA. This combination also produced highest fresh weight (5.4 g.) of callus per culture and produced friable green callus. The highest number of shoots (2.4) per explant was recorded on MS medium supplemented with 1.5 mg/1 BAP + 0.5 mg/1 IAA for internode segment. The maximum number of roots (5.1)per shoot was observed in the MS medium having 1.0 mg/1 IBA + 2.0 mg/1 NAA. The well Rooted plantlets were acclimatized and successfully established into soil in the natural condition where 60% plantlets were survived.

Keywords: *Dalbergia sissoo*, nodal segment, internodal segments, shoot tip, callus, embryo, regeneration.

Introduction

Dalbergia sissoo (2n = 20) belongs to the Family Fabaceae (Lindl 1836). About 10 species of *Dalbergia* found in this sub-continent, of which 5 species are available in Bangladesh. *D. sissoo* is timber yielding tree. It is a fast growing tree and is widely planted. Sissoo is one of the most useful multipurpose trees of Bangladesh. It plays an important role in environment conservation and ecosystem balance. The wood of this tree dries up easily and has a great demand for construction of high class furniture. It is also used for commercial plywood. The wood is excellent for fuel wood and charcoal. The bark, wood, root, leaf, juice and seed oil of the tree have many medicinal properties. Reported to be stimulant, sissoo is a folk remedy for excoriations, gonorrhea and skin ailments (Duke and Wain 1981).

Lack of disease free planting material and quality seed is greatly affecting the economy of many developing countries of the world. Large scale propagation of elite clones from hybrids or specific parental line through commercial propagation or somatic embryogenesis hold the promise of alleviating these problems. The process of micropropagation lies in the combination of rapid multiplication of elite clones plus pathogen controlled program. So far a limited works has been published in this field of study but it holds a great opportunity in future progress.

Sissoo is mostly propagated by seeds or by planting suckers. Conventional methods of planting have been described by a number of authors (Vidacovic 1968, Dhuria 1992 and Kulkarni and Takawale 2000). But the techniques are laborious and time consuming as far as production of large number of homogeneous plants are concerned. Clonal propagation of *Dalbergia* sp. cultures by suckers is seriously limited for its low multiplication rate. Clonal multiplication of sissoo, which is so important in forestry, is generally achieved through vegetative propagation. Most of the crop plants especially those propagated by seed or vegetative means are systematically infected with one or more pathogens. Furthermore, sissoo has been seriously

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affected in recent years by different fungal or insect pest causing diseases, such as die back, root and butt rot, leaf defoliation, leaf rolling, foliage rust and powdery mildew (Jackson 1987). The exchange of plant propagation material from country to country or continent to continent involves the possible dissemination of these diseases. The conservation of *Dalbergia* spp. genetic resources are also problematic since the diseased free species are not possible to conserve in field gene bank where maintenance cost is also high. In this experiment an easy protocol is described for indirect regeneration from nodal and internodal segment and shoot tip of sisoo.

Materials and Methods

The nodal segment, internodal segment and shoot tip of sissoo were used as experimental materials in this investigation. All explants were collected from Rajshahi University Campus, Rajshahi, Bangladesh and explants were surface sterilized with $HgCl_2$ solution (.1% w/v) for 10 minutes. For inoculation, the explants were cut into appropriate size cultured on MS medium (Murashige Skoog 1962). The PH of medium was 5.8.The cultures were incubated in a culture room at 27± 2°C with a photoperiod of 16 hours at 3000 lux light intensity provided by cool white fluorescent tubes. About 30 days after initiation, calli were rescued aseptically on a sterile petridish and were cut into convenient size by a sterile scalpel and these were inoculated to a freshly prepared MS medium supplemented with the same or different hormonal combinations either for the maintenance of callus for shoot regeneration and root induction.

The regenerated shoots were very carefully rescued from the culture bottles, placed on a sterile petridish and cut from the basal end of the shoots. Then each of the shoot was cultured on freshly prepared medium containing full strength of MS salt with different concentrations combinations of hormonal supplements for root induction.

Well developed plantlets were removed from culture bottle and planted in plastic pots containing a potting mixture of sand, compost and garden soil in the ratio of 1:1:1. The potted plantlets were covered by polythene bag to maintain suitable humidity. After 30-35 days of bagging, the plantlets were transplanted in natural condition, where 60% plants survived.

Result and Discussion

Callus induction and its subculture: Among three types of explants viz. *in vivo* nodal and internodal segments and shoot tips of *Dalbergia sissoo,* best callus induction was observed in nodal segment. It was observed 100% in MS medium supplemented with 2.0 mg/1 BAP + 0.5 mg/1 NAA. This combination also produced highest fresh weight of callus per culture and it was 5.4 g. The quickest average response of the explants took 6.1 days old culture in the same media compositions. The colour of callus was green and friable in nature in this case. The second best 90% of callus was found in 2.0 mg/1 BAP + 0.1 mg/1 IAA for internodal segments. The calli were friable in nature. Other type of explants viz. shoot tip showed relatively poor performance for callus induction (Table 1). According to Mamun (2000) internodal segments from forest tree *Albizia lebbeak* showed best results for callus induction (100% in MS medium containing with 2.0 mg/1 BAP + 0.2 mg/1 NAA and 2.0 mg/l BAP + 0.5 mg/1 NAA) in compared to nodal segments and in case of *Dalbergia sissoo* nodal segments collected from field grown mature tree were the best for callus induction (90.90% in 2.0 mg BAP + 0.2 mg/1 NAA media compositions). But according to Pattnaik *et al.* (2000) hypocotyl segments were most responsive in callus induction. The present findings is almost simmilar to that of Mamun's (2000) result.

Table 1 Effect of different concentrations and combinations of cytokinins and auxins in MS medium for induction of callus from the *in vivo* nodal and internodal segments and shoot tips of the stumps of *Dalbergia sissoo.*

Growth regulators	Concentration of growth regulators mg/l	Nodal segment			Internodal segment			Shoot tip		
		Days to response of callus induction	% of explants responded	Type of callus	Days to response of callus induction	% of explants responded	Type of callus	Days to response of callus induction	% of explants responded	Type of callus
	0.5	18.1	10	Gy, L	-	-	-	20.1	10	Gy, L
	1.0	15.0	20	Gy, L	16.1	10	L Gn, F	17.2	20	L Gy, C
BAP	1.5	14.2	40	L. Gy, C	15.3	30	L Gn, F	16.3	30	L Gn, F
	2.0	12.3	50	L. Gn, F	13.2	40	L Gn, F	14.4	40	L Gy, C
	2.5	13.5	30	L. Gn, C	13.5	20	L Gn, C	15.6	20	L Gy, C
	1.5+0.5	10.2	60	D Gn, F	11.3	50	D Gn, F	12.9	40	D Gn, F
	1.5+1.0	8.1	60	Gn, F	9.2	40	D Gn, F	10.4	50	D Gn, C
BAP+NAA	2.0+0.1	10.3	50	Gn, F	11.1	40	Gn, F	12.1	30	Gn, F
	2.0+0.5	9.1	100	Gn, F	10.6	60	Gn, F	11.1	50	Gn, F
	2.0+1.0	8.6	80	Gn, C	9..9	80	Gn, C	10.3	70	Gn, C
	1.5+0.5	12.3	30	L Gn, F	13.3	20	Gn, F	14.2	10	L Gn, F
	1.5+1.0	8.6	50	L Gn, F	9.3	40	Gn, F	10.1	30	L Gn, C
BAP + IBA	2.0+0.1	8.3	60	Gn, F	9.2	50	Gn, F	11.4	40	Gn, F
	2.0+0.5	10.1	50	Gn, F	11.1	40	Gn, F	12.8	30	Gn, F
	2.0+1.0	7.2	80	Gn, F	8.6	70	Gn, F	9.9	60	Gn, F
	1.5+0.5	9.6	70	Gn, F	10.2	60	Gn, F	12.1	60	Gn, F
	1.5+1.0	10.2	60	Gn, F	11.3	50	Gn, F	13.3	40	Gn, C
BAP+IAA	2.0+0.1	6.1	90	Gn, F	7.9	90	Gn, F	9.0	80	Gn, F
	2.0+0.5	7.6	80	Gn, F	8.8	70	Gn, F	10.5	60	Gn, F
	2.0+1.0	9.1	70	Gn, F	10.6	60	Gn, F	11.3	40	Gn, C
	0.5	19.4	10	Cr, L	20.3	10	Cr, L	21.1	10	Cr, C
	1.0	17.2	30	Cr, C	18.2	20	Cr, C	19.2	20	Cr, C
KIN	1.5	12.4	40	Cr, C	14.9	30	Cr, C	15.3	30	Cr, C
	2.0	13.2	50	Cr, C	15.8	20	Cr, C	16.6	20	Cr, C
	2.5	15.3	20	Cr, C	17.6	10	Cr, L	-	-	Cr, C
	1.5+0.5	12.3	50	L Cr, F	13.3	40	L Cr, F	14.2	30	L Cr, F
	1.5+1.0	11.1	40	L Cr, F	12.6	30	L Cr, F	13.3	10	L Cr, F
KIN+NAA	2.0+0.1	15.3	20	L Cr, F	-	-	-	19.5	10	L Cr, F
	2.0+0.5	15.2	30	L Cr, F	-	-	-	18.2	10	L Cr, F
	2.0+1.0	14.2	30	L Cr, F	15.3	20	L Cr, C	17.3	20	L Cr, F
	1.5+0.5	15.2	10	L Cr, C	17.2	10	L Cr, C	18.6	10	L Cr, C
	1.5+1.0	14.4	30	L Cr, C	15.1	20	L Cr, C	16.7	20	L Cr, L
KIN+IBA	2.0+0.1	14.2	10	L Cr, C	15.3	10	L Cr, C	-	-	-
	2.0+0.5	16.2	10	L Cr, C	-	-	-	-	-	-
	2.0+1.0	15.3	20	L Cr, L	16.3	20	L Cr, L	17.1	10	L Cr, C
	1.5+0.5	11.2	30	Gr, F	14.2	20	Gn, F	16.3	10	Gn, F
	1.5+1.0	13.3	20	Gr, C	13.1	10	Gn, C	14.1	10	Gn, F
KIN+IAA	2.0+0.1	12.4	30	Gr, F	16.3	20	Gn, F	18.1	20	Gn, F
	2.0+0.5	14.1	20	Gr, F	-	-	-	21.3	10	Gn, F
	2.0+1.0	15.2	10	Gr, F	-	-	-	20.5	10	Gn, F

Gy=Gray, L=Loose, C=Compact, Gn=Green, F=Friable, Cr=Cream

Indirect shoot regeneration through callus: Auxin-cytokinin balance was necessary for the regeneration of callus into plantlets in culture. Regeneration of complete plants from callus is still presented as a big problem particularly in woody plant. In the present investigation, a huge amount of calli obtained from three types of explants of sissoo were able to regenerate when the callus was taken from recaptured MS media

supplemented with 2.0 mg/1 BAP + 0.5 mg/1 NAA or 2.0 mg/1 BAP + 0.5 mg/1 IAA hormonal combination. Relatively a good number of shoot regeneration observed on callus population derived from internodal segments of the *in vivo* grown stumps. *In vivo* nodal segments derived calli were also good in regeneration but the number of regenerated shoot was less than those of calli derived from internodal segments. Calli production by *in vivo* shoot tips were the poorest in respect of shoot regeneration as well as number of regenerated shoots. Among the three types of calli such as friable, loose and compact only the friable green calli were able to regenerate shoots. All the three types of explants derived callus, further callus induction and shoot regeneration occurred when higher concentrations of BAP with NAA, IBA and IAA were used and less callus induction and shoot regeneration was observed in media with different concentrations of BAP alone and KIN with NAA and IAA in higher concentrations. But in both cases, lower concentrations failed to regenerate shoots. According to Chand *et al*. (2005) shoot regeneration occurred when calli clumps were transferred to MS medium enriched with BAP + NAA combination and maximum response (45%) for shoot regeneration was achieved when calli clumps were transferred to MS medium supplemented with 8.88 mM BAP and 1.34 mM NAA. This result supports the present findings.

Different concentration of BAP singly, KIN singly and their combinations with auxins showed various degree of regeneration. In case of *in vivo* internodal segments derived callus, the highest 2.4 number of shoots were produced in 1.5 mg/l BAP + 0.5 mg/1 IAA media combination (Table 2) followed by BAP + IBA and BAP + NAA combinations respectively. In case of *in vivo* nodal segments derived callus and *in vivo* shoot tip derived callus, maximum 2.2 and 1.8 number of shoots were regenerated in 1.5 mg/1 BAP + 0.5 mg/1 IAA, and 2.0 mg/1 BAP + 0.1 mg/1 NAA media combinations respectively (Table 2). Pattnaik *et al*. (2000) found that 71% of cultures exhibited shoot bud differentiation on a MS medium containing 2.0-3.0 mg l^{-1} (8.9-13.3 µM) BAP and 0.5 mg /l (2.7 µM) NAA. This result is partially similar to the present studys when shoot tips derived callus were used.

Induction of root: Induction of roots from the shoot cutting with vascular continuity results in the development of complete plants. Micro propagation is meaningless without successful establishment of plantlets in the soil. For this purpose, shoots were treated with basal MS media with full and half strength of macro and micro nutrient supplemented with various types of auxins (NAA, IAA and IBA) and their combination with each other.

The micro shoots of the woody legume sissoo were used for root induction in MS and ½ MS media supplemented with different concentrations of IBA, IAA and NAA separately and in combinations. IBA with NAA is the best suited media for root initiation in both for MS and ½ MS medium (Table 3) which was followed by IBA with IAA, IBA, NAA and IAA in case of MS medium and, IBA with IAA, IBA, NAA, and IAA singly in case of ½ MS medium. In this experiment, MS medium is found to be more efficient than ½ MS medium. The highest 90% micro shoots initiated root was obtained in media having MS + 1.0 mg/l IBA + 2.0 mg/l NAA combination and the highest mean number of root was 5.1 also found in the same media composition. The quickest average response for the roots initiation of the micro shoots took 12.6 days old culture in media supplemented with MS + 1.0 mg/l IBA + 2.0 mg/l NAA and 2.0 mg/l IAA (Table 3).

Table 2. Effect of subculturing of callus of *Dalbergia sissoo* in different concentrations and combinations of growth regulators in MS medium on shoot formation.

Supplements in precultures mg/l	Supplements mg/l	Callus derived from nodal segments		Callus derived from inter – nodal segments		Callus derived from shoot tips	
		No of Shoot /culture	Survival of callus	No of Shoots /culture	Survival of callus	No of Shoots/culture	Survival of callus
2BAP+0.5NAA	BAP						
	1.0	-	+	-	+	-	-
	1.5	1.1	+	1.3	+	0.5	+
	2.0	0.7	+	0.6	+	-	+
	2.5	-	+	-	+	-	+
	BAP+NAA						
	1.5 + 0.5	1.5	+	1.6	+	1.1	+
	1.5 + 1.0	0.6	+	0.5	+	-	+
	2.0 + 0.1	1.1	+	1.0	+	0.6	+
	2.0 + 0.5	1.3	+	1.4	+	1.0	+
	BAP+IBA						
	1.5 + 0.1	1.5	+	1.6	+	-	+
	1.5 + 0.5	1.3	+	1.2	+	0.7	+
	1.5 + 1.0	1.1	+	0.9	+	-	+
	2.0 + 0.1	2.1	+	2.0	+	1.2	+
	BAP+IAA						
	1.5 + 0.1	1.2	+	1.3	+	1.2	+
	1.5 + 0.5	2.2	+	2.4	+	1.4	+
	1.5 + 1.0	1.1	+	0.9	+	-	+
	2.0 + 0.1	1.6	+	1.5	+	1.1	+
2BAP+ 0.5IAA	BAP						
	0.5	-	+	-	+	-	+
	1.0	-	+	-	+	-	+
	1.5	1.2	+	1.3	+	1.0	+
	2.0	0.8	+	0.6	+	-	+
	BAP+NAA						
	1.5 + 0.1	0.9	+	1.0	+	-	+
	1.5 + 0.5	1.5	+	1.4	+	1.0	+
	1.5 + 1.0	0.7	+	0.8	+	0.4	+
	2.0 + 0.1	1.0	+	1.1	+	0.2	+
	BAP+IBA						
	1.5 + 0.1	1.4	+	1.5	+	1.4	+
	1.5 + 0.5	1.3	+	1.4	+	1.0	+
	1.5 + 1.0	0.9	+	0.8	+	-	+
	2.0 + 0.1	1.9	+	2.0	+	1.8	+

Table 3. Effect of different concentrations and combinations of auxins in MS and ½ MS medium for induction of root from *in vitro* grown micro cutting of multiple shoots of *Dalbergia sissoo*.

Supplements mg/l	*In vitro* grown micro cutting of multiple shoots in MS medium			*In vitro* grown micro cutting of multiple shoots in ½ MS medium		
	% of explants induced root development	Time Taken to initiate root	Mean number of root per explant	% of explants induced root development	Time Taken to initiate root	Mean number of root per explant
IBA						
1.0	40	17.1	2.8	30	22.2	1.3
2.0	50	15.3	3.1	40	18.3	2.6
3.0	30	20.2	1.6	20	22.1	1.1
4.0	20	21.4	1.2	-	-	-
IAA						
1.0	-	-	-	-	-	-
2.0	10	25.1	1.0	20	24.6	1.0
3.0	30	20.6	1.2	30	21.7	1.2
4.0	-	-	-	10	25.2	1.0
NAA						
1.0	40	18.5	2.7	60	16.3	4.1
2.0	30	19.3	1.5	40	17.2	2.7
3.0	20	21.2	1.3	30	19.7	1.2
4.0	-	-	-	20	24.1	1.0
IBA + IAA						
1.0 + 2.0	20	23.7	1.0	20	27.3	1.0
2.0 + 1.0	40	17.9	2.9	40	19.7	2.5
2.0 + 2.0	60	14.4	3.6	50	18.2	2.9
3.0 + 1.0	20	21.3	1.2	10	26.5	1.0
3.0 + 2.0	30	19.2	1.4	20	21.9	1.3
4.0 + 1.0	-	-	-	-	-	-
IBA + NAA						
1.0 + 1.0	40	16.1	3.1	30	18.5	1.4
1.0 + 2.0	90	12.6	5.1	70	14.1	4.7
2.0 + 1.0	30	18.9	1.5	20	22.3	1.3
2.0 + 2.0	60	13.7	3.8	50	17.7	3.0
3.0 + 1.0	20	20.3	1.2	20	25.2	1.0
3.0 + 2.0	30	19.2	1.4	20	23.9	1.1
4.0 + 1.0	20	22.4	1.0	-	-	-

According to Mamun (2000) the highest 90.90% of micro shoots of woody legumes *A. catechu* and *A. lebbeck* regenerated roots in media containing with MS + 1.0 mg/l IBA and MS + 1.0 mg/l IBA respectively. But maximum 81.81% of micro shoots of *D. sissoo* regenerated roots on media having MS+3.0 mg/l IBA and initiation of root from micro shoots of *D. sissoo* took 10 days only which is a good agreement to my findings for MS medium.

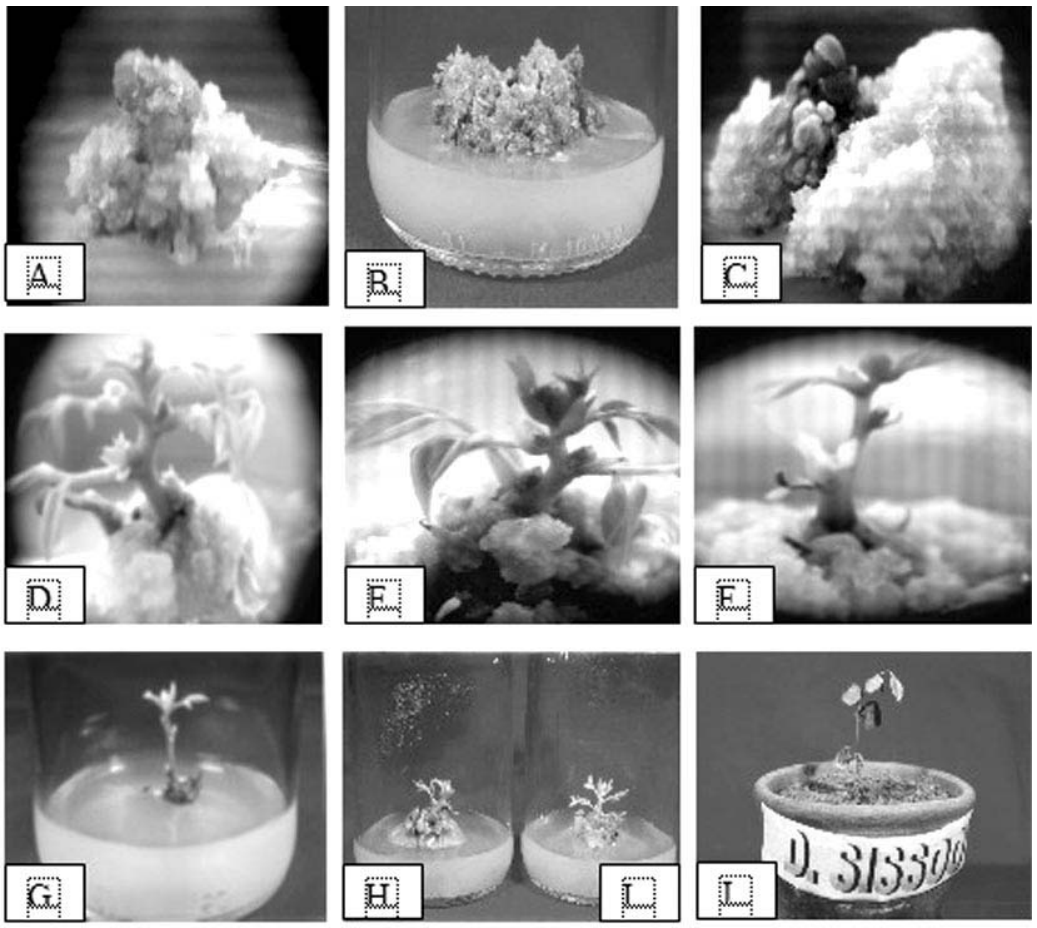

Fig.1. Callus induction and Shoot and root proliferation from *in vivo* nodal and internodal segments and shoot tip of sissoo (*Dalbergia sissoo*). A, B and C: Callus induction from *in vivo* nodal segment, internodal segment and shoot tip respectively. D, E and F: shoot proliferation from *in vivo* nodal segment, internodal segment and shoot tip derived callli respectively., G, H and I: Root formation from nodal segment, internodal segment and shoot tip derived shoot on MS medium. and J: Aclimatized plant

But in case of ½ MS medium the highest 70% of micro shoots initiated roots in 1.0 mg/l IBA + 2.0 mg/l NAA, the highest mean number of root were 4.7 and the quickest average response for the root initiation took 14.1 days old culture in the same medium composition (Table 3). According to Joshi *et al*. (2003), maximum number of roots per plantlet (4.47) was observed in ½ MS supplemented with (1.0 mg/l) IBA within 18 days. This conclusion is little bit dissimilar to the present result but similar to that of Gill *et al*. (1997). They found that the elongated shoots were rooted successfully through culturing on half strength MS medium supplemented with IBA (1.0 mg/l) and NAA (0.2 mg/l). Plantlets of sissoo was established in polybag containing mixture of garden soil, compost and sand in the ratios of 1:1:1. These were subsequently acclimatized to out door conditions. The survival rate of the plant was 60%. Similar survival rates were obtained by Roy and De (1986) in *Calotropis gigantea*, Reza (1994) in *Albizia lebbeck*, Obidulla (1995) in *Dalbergia sissoo* and Mamun in *D. sissoo* (2000).

In conclusion, we report an efficient and easy to handle protocol for micropropagation of sissoo. This protocol provides a successful and rapid technique that can be used for ex-situ conservation.

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