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Article

Shoots of *Agave nussaviorum* García-Mend. rooted by varying inorganic salts, IBA and incubation

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Abstract

In Oaxaca, Mexico, populations of *Agave nussaviorum* are declining and micropropagation is proposed in addition to the use of conventional methods. This work aimed to evaluate the effect of the concentration of inorganic salts and indole-butyric acid in the culture medium, as well as the incubation environment, to root shoots *in vitro*. *In vitro* cultured shoots were transferred for rooting to nine culture medium variants that differed in the concentration of inorganic salts of the culture medium (60, 80 and 100%) and indole-butyric acid (0, 0.5 and 1 mg L^{-1}) in a factorial design with nine treatments. For 28 days, all cultures were incubated in the laboratory with LED lighting and the percentage of shoots with roots was evaluated. From day 29 onwards, half of the cultures in each variant of culture medium were separated to incubate them for 21 days exposed to solar radiation reduced by 40% by shade mesh in a greenhouse, while the rest continued in incubation with LED lamps in the laboratory. At the end of the first period, the highest percentage, 83.3% of shoots with roots, was in the culture medium with 60% inorganic salts and 0.5 mg L^{-1} of indole-butyric acid. At the end of the second period, all shoots had adventitious roots under the following conditions: culture medium with 60% salts and 0.5 or 1 mg L^{-1} indole-butyric acid incubated in the laboratory; culture medium with 60% inorganic salts and 1 mg L^{-1} indole-butyric acid incubated in a greenhouse; culture medium with 100% inorganic salts and 0.5 mg L^{-1} indole-butyric acid incubated in a greenhouse.

Keywords:

Agave nussaviorum, adventitious roots, micropropagation.

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Introduction

The species *Agave angustifolia* (Cruz-García *et al*., 2013), *A. tequilana* (Arzate-Fernández *et al*., 2016), *A. potatorum* (Molina-Guerrero *et al*., 2007), *A. americana* var. oaxacensis (Pérez-Santiago *et al*., 2014; Cruz-García *et al*., 2017) and *A. nussaviorum* are used to make distilled beverages. During 2021, in Oaxaca, there were 10 839.57 hectares of plantations and 2 992.19 ha were harvested (SIAP, 2021). There are no published data on the magnitude of wild agaves collection.

A. nussaviorum, which is known as papalomé in the Mixteca region of Oaxaca, is used for food, in traditional medicine (García-Mendoza, 2010) and to make mezcal. This species is collected from wild populations; therefore, its propagation is proposed to establish plantations. Plant tissue culture is applied in agaves to produce a greater number of plants in complement to conventional propagation procedures. There are records of the micropropagation of *Agave tequilana* (Valenzuela-Sánchez *et al*., 2006), *A. angustifolia* (Ríos-Ramírez *et al*., 2017), *A. fourcroydes* (Madrigal *et al*., 1990), *A. americana* var. oaxacensis (Enríquez-Valle *et al*., 2013) and *A. salmiana* (Silos-Espino *et al*., 2007).

In vitro propagation comprises five stages: a) preparation of the mother plants under hygienic conditions; b) establishment of aseptic cultures; c) multiplication of propagules; d) shoot rooting and e) transplantation into containers with substrate for acclimatization in a greenhouse (George and Debergh, 2008). For stages b, c and d, it is necessary to determine the conditions of mineral salts, carbohydrates, vitamins, growth regulators in the culture medium and the incubation environment (Puente-Garza *et al*., 2017).

The quality of the micropropagated plant influences the success of its acclimatization. In relation to stage d, the ability of tissues to form adventitious roots depends on endogenous factors (genotype, physiological condition, etc.) and exogenous factors (substrate, relative humidity, temperature, irradiance). In order for the shoots to develop roots, they are established in culture media (CM) without growth regulators (GR) or with an auxin-type GR.

During the *in vitro* rooting of *A. angustifolia* (Enríquez-Valle *et al*., 2005) and *A. potatorum* (Bautista-Castellanos *et al*., 2020) shoots, when established in CM with auxins, they develop more roots in less time. In both species, shoots in CM with the MS (Murashige and Skoog, 1962) mineral salts at 75% had a better rooting response compared to shoots in CM with the MS salts at 100%. Miguel-Luna *et al*. (2013) reported that shoots of *A. americana* var. oaxacensis established in CM with 66% MS salts formed 7.3 adventitious roots on average, a number 15% higher than the roots formed in shoots established in CM with 100% inorganic salts.

The concentration of solutes in the CM, mainly inorganic salts and sucrose, determines its osmotic potential (Ψ), which influences the formation, quantity, and growth of roots in shoots since levels lower than -0.3 MPa inhibit root formation (Cárdenas-Lara and Villegas-Monter, 2002).

In vitro propagated plants have thin leaves with herbaceous consistency, with little cuticle development, inefficient roots, high stomatal density and stomata with little control of opening and closing. When these plants are extracted from the *in vitro* culture to an environment with lower relative humidity, they have difficulty controlling water loss, being exposed to excessive wilting, in addition to presenting low photosynthetic activity (Pospíšilová *et al*., 2000; Pospíšilová *et al*., 2007; Sosa-Castillo *et al*., 2014).

Micropropagated plants of *A. americana* var. oaxacensis, during their acclimatization in greenhouses, replaced several leaves that they had formed during their *in vitro* culture with new leaves with different characteristics related to their adjustment to the *ex vitro* environment (Cruz-García *et al*., 2017). Micropropagated agaves must have morphological and physiological characteristics that, when large quantities of plants are extracted from the CM, make it easier for them to adapt to the *ex vitro* environment, achieving high levels of survival and growth (Miguel-Luna *et al*., 2013; Enríquez-Valle *et al*., 2016).

Authors such as Pospíšilová *et al*. (1999) described that plants micropropagated during the first three to four weeks of acclimatization stop their growth and even reduce their size due to organ

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senescence. It is argued that these leaves die because they do not have the capacity to make changes related to their adjustment to the *ex vitro* environment. It is desirable that the plant grown *in vitro* develops organs that persist and are efficiently functional during and after acclimatization. In the micropropagation of Musa, Texeira-da Silva *et al*. (2005) subjected *in vitro* cultures of Musa and Cymbidium in the rooting stage to a process of pre-acclimatization or pre-adaptation by exposing them to decreased solar radiation by partial shade, environmental conditions with lower relative humidity. The research aimed to evaluate the effect of the concentration of mineral salts of the MS medium and IBA auxin in the culture medium on the rooting of *A. nussaviorum* shoots, as well as the incubation environment on the morphological characteristics of the plants.

Materials and methods

Obtaining shoots for rooting

The work was conducted in the plant tissue culture laboratory and in a greenhouse at the Technological Institute of the Valley of Oaxaca in the municipality of Santa Cruz Xoxocotlán, Oaxaca. *In vitro* cultures were obtained from clusters of *A. nussaviorum* shoots heterogeneous in size in the propagule multiplication stage.

From each cluster, shoots with 3 to 4.5 cm length of the largest leaf were selected, separated individually and established in 160 cm^3 bottles containing 20 ml of one of the nine variants of sterilized CM with gel consistency, prepared with: distilled water, 1 mg L^{-1} thiamine-HCL, 25 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol; MS (Murashige and Skoog, 1962) mineral salts in three different dilutions (60, 80 and 100%), indole-3-butyric acid (IBA) in three different concentrations (0, 0.5 and 1 mg L^{-1}), pH adjusted to 5.8 before adding 5.7 g L^{-1} of agar. Two shoots were established in each culture bottle, the lid was placed, and the bottle was sealed with adhesive polyethylene.

Incubation conditions

During the first 29 days of the rooting stage, all cultures were incubated in the laboratory, exposed to LED lighting at 35 µmol m^{-2} s^{-1} in photoperiods of 16 h and 8 h of darkness, temperature in the range of 15-28 °C. After that time, half of the cultures of each variant of CM were transferred to a greenhouse environment to be exposed for the remaining 21 days to lighting with solar radiation reduced by means of a double layer of 40% shade mesh, while the other half of the cultures remained in the laboratory with LED lighting.

When the shoots were established in the rooting medium, data on height and number of leaves were taken and the shoots that had roots and the number of adventitious roots were recorded daily during the 50 days of this stage. At the end of this stage, the plants were extracted from the culture medium and the number of unfurled leaves, the length and width of the largest leaf, stem diameter, and number of roots were recorded.

The experiment was established according to a completely randomized design, which, in the first 29 days of this stage, had a 3 × 3 factorial treatment arrangement, three levels of the mineral salt concentration factor, three levels of the IBA concentration factor and had nine treatments for days 30 to 50 of the rooting stage, the treatment arrangement was factorial of 3 × 3 × 2, which included the levels of the aforementioned factors and the incubation environment factor with two levels (laboratory and greenhouse), so there were 18 treatments.

The experimental unit was a shoot and there were 12 replications per treatment. To compare the percentages of shoots that formed roots, the experimental unit was a group of four shoots and there were six and three replications per treatment on days 29 and 50 of the experiment, respectively. The data in percentage for analysis were transformed into arcsine. The data were subjected to analysis of variance and comparison of means using Tukey's test ($\alpha \leq 0.05$).

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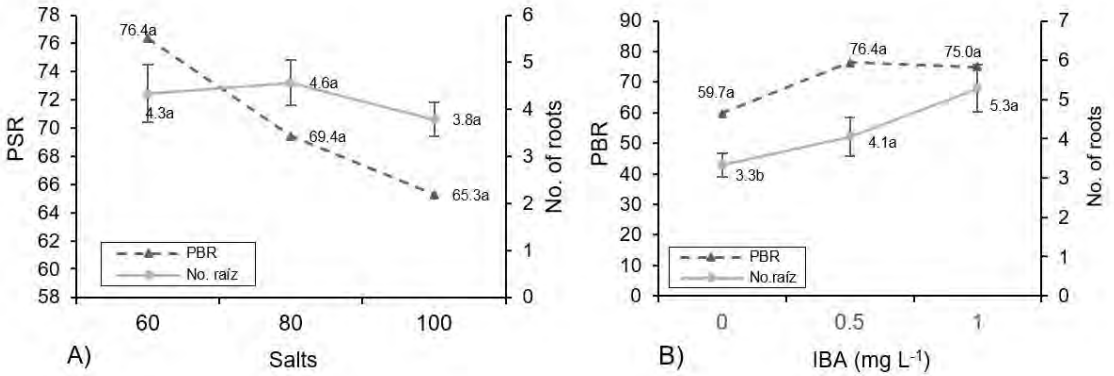
Results and discussion

Rooting stage under laboratory conditions

At the beginning of the rooting stage, the shoots of the different groups had 3.4 to 4.5 leaves and were 3.6 to 4.4 cm tall, which, in each case, were not significantly different magnitudes, indicating that the plant material that was distributed in the different treatments was relatively homogeneous. Eight days after the shoots were established in the culture medium, the first shoots with adventitious roots were observed. Of the groups of shoots that were established in the different variants of CM, after 29 days of incubation in laboratory conditions, between 58.33% and 83.33% of them had roots (Figure 1 A).

Figure 1. In the first 29 days of the rooting stage, the percentage of shoots (dashed line) of *A. nussaviorum* that formed roots (PSR) and number of roots formed (solid line) as a function of the main factors, mineral salts (A) and IBA (B).



Shoots in CM with 80% mineral salts (MS) and 1 mg L^{-1} IBA formed 7.5 adventitious roots, three times the number of roots of shoots in CM with 100% MS without IBA. The lowest percentages of shoots with roots were those in CM with the 100% MS mineral salts and without IBA. Ordering the variables according to factor levels, the highest percentage of shoots with roots (PSR) was in CM with 60% MS salts and 0.5 mg L^{-1} IBA, while the shoots with the highest number of roots were in CM with 60% MS salts and 1 mg L^{-1} of IBA (Figure 1B).

In some shoots, roots were observed at eight days, while in other shoots, roots were observed up to 50 days. In other species, *A. tequilana* (Monja-Mio *et al*., 2020), *A. angustifolia* in CM with 50% MS inorganic salts and 0.025 mg L^{-1} of 2,4-D (Sánchez *et al*., 2020) and *A. fourcroydes* (Garriga *et al*., 2010) are reported to have formed roots in 100% of shoots. Likewise, Bautista-Castellanos *et al*. (2020) report that, in *Agave potatorum* Zuc shoots, adventitious roots were observed between 13 and 37 days of incubation.

Regarding the shoots that were established in CM with IBA and inorganic salts at 75% concentration, 95% of them formed adventitious roots. The data of the present study coincides with previous work in that shoots had a better adventitious root formation response when they were established in CM with low concentrations (60 or 80%) of mineral salts.

Second rooting period

After 50 days of incubation in the rooting stage, analyses of variance (Table 1) showed that mineral salt concentrations had significant effects ($p \leq 0.03$) on the number of leaves; levels of indole-3-butyric acid (IBA) concentration factor had significant effects on root number ($p \leq 0.01$) and root length ($p \leq 0.03$), while the levels of the incubation condition (IC) factor had significant effects on plant height ($p \leq 0.0002$).

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Table 1. Analysis of variance of the morphological characteristics of *A. nussaviorum* plants 50 days after establishing shoots in the rooting stage.

Source of variation	DF	Mean squares and significance					
		PSR	FNL	FNR	FHEI	RL	SD
Treat	17	496.7ns	3.14*	20.97ns	5.06ns	7.12ns	1.66ns
Salts	2	816.6ns	6.68*	13.72ns	0.44ns	1.46ns	2.06ns
IBA	2	516.6ns	1.84ns	67.44**	3.85ns	16.55*	0.91ns
IC	1	150ns	5.35ns	28.38ns	45.55**	5.8ns	5.44ns
Salts×IBA	4	260.4ns	1.4ns	9.87ns	1.23ns	5.52ns	1.45ns
Salts×IC	2	950ns	0.22ns	3.21ns	4.84ns	6.05ns	3.88ns
IBA×IC	2	0ns	0.36ns	8.5ns	1.78ns	6.06ns	0.85ns
Salts×IBA×IC	4	668.7ns	6.07**	25.74ns	3.41ns	8.24ns	0.37ns
Error	198	295.83	1.87	16.49ns	3.15ns	4.97ns	1.63ns
Total	215						
CV (%)			29.31	66.69	26.92	69.81	24.45

SV= sources of variation; DF= degrees of freedom; CV= coefficient of variation; IBA= indole-butyric acid; IC= incubation condition; PSR= percentage of shoots with roots (data transformed to arcsine); FNL= final number of leaves; FNR= final number of roots; RL= root length; FHEI=final height; SD= stem diameter; ns= not significant; *= significant (p> 0.05); **= highly significant (p> 0.01).

The interaction of mineral salts-IBA-incubation conditions had a significant effect (p# 0.01) on the number of leaves. Aguilar-Jiménez and Rodríguez de la O (2018) reported that shoots of *A. marmorata* grown in 100% MS CM and with 0.3 or 10 mg L^{-1} of IAA formed 4.1 and 4.9 adventitious roots on average. In contrast, shoots in CM with 3 or 10 mg L^{-1} of IAA formed roots with 8.2 and 9.3 cm in length, respectively, highlighting the importance of the type and concentration of auxin.

Auxins induce the formation and development of roots in shoots, and the amount and type of auxin and the response of the explant depends on the species to be worked on (George *et al*., 2008). The use of IBA in culture media has been shown to be efficient in inducing the rooting of shoots of *A. potatorum* (Bautista-Castellanos *et al*., 2020), *A. angustifolia* (Enríquez-Valle *et al*., 2005) and *Agave americana* (Miguel-Luna *et al*., 2013).

At the end of the shoot rooting stage, plants had 3.7 to 5.8 leaves, 2.7 to 7.9 roots, 1.2 to 4.9 mm in root length, 5.6 to 7.9 cm in height, and 4.7 to 5.8 mm stem diameter (Table 2). All shoots incubated in the laboratory in culture media with 60% MS salts with 0.5 or 1 mg L^{-1} IBA had roots.

Table 2. Characteristics of *A. nussaviorum* plants at the end of 50 days of shoot rooting under different culture and incubation conditions.

IS	IBA (mg L^{-1})	IC	SR (%)	FNL	FNR	FHEI (cm)	RL (cm)	SD (mm)
60	0	L	75a	5.8 ±0.5a	3.8 ±0.8a	7.9 ±0.8a	2.8 ±0.9ab	5.7 ±0.6a
60	0	Gr	91.7a	4.8 ±0.4ab	5.5 ±1.2a	5.9 ±0.3a	2.9 ±0.5ab	5.8 ±0.4a
60	0.5	L	100a	4.7 ±0.4ab	7.6 ±1.8a	6.6 ±0.6a	2.6 ±0.3ab	5.3 ±0.3a
60	0.5	Gr	75a	5.3 ±0.4ab	4.5 ± 1a	6.3 ±0.3a	1.5 ±0.3b	5.3 ±0.3a
60	1	L	100a	4.1 ±0.3ab	6.5 ±1.9a	7.3 ±0.7a	4.1 ±1.7ab	5.2 ±0.2a
60	1	Gr	100a	5.1 ±0.3ab	7.9 ±1.2a	6.1 ±0.4a	3.3 ±0.5ab	4.9 ±0.3a
80	0	L	75.9a	4.3 ±0.3ab	5.4 ±0.8a	7.1 ±0.4a	4.9 ±0.8a	5 ±0.3a
80	0	Gr	66.4a	5.4 ±0.5ab	3 ±1.3a	6.7 ±0.5a	1.8 ±0.5ab	5.5 ±0.5a

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IS	IBA (mg L^{-1})	IC	SR (%)	FNL	FNR	FHEI (cm)	RL (cm)	SD (mm)
80	0.5	L	91.7a	5 ±0.3ab	3.3 ±0.5a	6.5 ±0.5a	2.5 ±0.5ab	5.3 ±0.3a
80	0.5	Gr	91.7a	4.8 ±0.3ab	6.1 ±1.7a	6.3 ±0.6a	2.5 ±0.4ab	5.2 ±0.3a
80	1	L	44.5a	4.3 ±0.3ab	5.1 ±1.4 a	6.4 ±0.5 a	2.3 ±0.7 ab	5.6 ±0.3a
80	1	Gr	75a	4.6 ±0.7ab	6.1 ±1.7a	6.1 ±0.6a	2.5 ±0.6ab	5.2 ±0.5a
100	0	L	41.7a	4.2 ±0.4ab	3.6 ±0.7a	6.9 ±0.8a	1.4 ±0.5b	5.5 ±0.5a
100	0	Gr	83.3a	4.6 ±0.4ab	3.6 ±0.7a	6.5 ±0.3a	2.5 ±0.6ab	4.7 ±0.4a
100	0.5	L	66.7a	3.7 ±0.3b	2.7 ±0.6a	6.9 ±0.5a	1.2 ±0.3b	5.3 ±0.4a
100	0.5	Gr	100a	4.7 ±0.3ab	7.3 ±0.9a	5.6 ±0.5a	3.2 ±0.2ab	4.4 ±0.2a
100	1	L	66.7a	4.7 ±0.3ab	4.9 ±1.3a	7.9 ±0.4a	2.9 ±0.9ab	5.6 ±0.3a
100	1	Gr	83.3a	4.3 ±0.5ab	5.5 ±1.1a	5.8 ±0.3a	2.6 ±0.4ab	4.8 ±0.2a

IS= concentration of inorganic salts; IBA= indole-butyric acid; IC= incubation condition; SR= shoots with roots (%); L= laboratory; Gr= greenhouse; FNL= final number of leaves; FNR= final number of roots; FHEI= final height; RL= root length; SD= stem diameter; in each column, means with the same letter are not significantly different (Tukey, 0.05). Averages ± standard error are shown.

All shoots incubated in greenhouses, which were in CM with 60% MS salts and 1 mg L^{-1} IBA or in CM with 100% MS salts and 0.5 mg L^{-1} IBA, formed adventitious roots. Enríquez-Valle *et al*. (2016) report that 93.6% of *A. potatorum* shoots established in CM with 50% MS mineral salts and 1 mg L^{-1} IBA and incubated under fluorescent lighting in the laboratory and solar radiation in the greenhouse had formed roots at 28 days of incubation.

During incubation, the quality of light provided by artificial lighting influences physiological and morphological characteristics. In the present work, the light intensity provided by the LED lamps was 35 µmol m^{-2} s^{-1}, without variation; in contrast, in the incubated cultures exposed to solar radiation decreased by shade mesh, during the second half of the rooting period, the irradiance was very variable that, at noon, it was at 400 µmol m^{-2} s^{-1}, much higher light intensity and with a wider spectrum of wavelengths compared to the artificial lighting provided in the laboratory.

In the plants *in vitro* in the pre-acclimatization stage and incubated in the greenhouse, after a week in this condition, their leaves changed coloration on the underside, they went from a homogeneous green tone to areas with reddish coloration that turned purple after the three weeks of pre-acclimatization; likewise the leaf blades were unfolded, slightly concave towards the beam, had thorns at the edges, and showed a more rigid appearance.

On the other hand, the plants that remained in the laboratory retained their leaves with a more homogeneous green coloration and they were larger with few thorns on the edge of the leaf blade. According to Peng *et al*. (2008), the reddish and purple coloration is due to the presence of anthocyanins, which are secondary metabolites that protect the leaves and the photosynthetic apparatus from damage caused by high light intensity.

The characteristics of plants due to the *in vitro* environment in which they are obtained could have consequences in the later stage of acclimatization and affect their adaptation in an *ex vitro* environment. Teixeira-da Silva *et al*. (2005) describe that, during the *in vitro* rooting of Musa shoots, they were subjected to pre-acclimatization conditions and the plants thus obtained developed greater vigor, pigmentation, photosynthetic activity, and waxy cuticle development.

Plants with these characteristics, when extracted from *in vitro* culture and established in containers with substrate and *ex vitro* environment, adapt better than plants from *in vitro* cultures incubated all the time in the laboratory with artificial lighting. Souza *et al*. (2021) describe that, in the micropropagation of *Cattleya warneri* T. Moore, *in vitro* cultures incubated in the laboratory had leaves that were 30% less thick compared to plants from cultures incubated in the greenhouse.

In *in vitro* cultures of *Vitis vinifera* L. (Li *et al*., 2017) that were exposed to lighting of different wavelengths: white, blue, green, and red, it was shown that plants express different groups of genes that affect their physiological performance and morphology. Borges *et al*. (2011) research reported that plants of *Dendranthema grandiflora* Tzevele cv. Rage micropropagated from *in vitro* cultures

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exposed to solar radiation in the pre-acclimatization stage presented thicker leaves, greater leaf area, high stomatal density and more developed roots, and improved their photosynthetic capacity.

In the present work, *Agave nussaviorum* plants that were obtained in culture media in which the concentration of mineral salts was reduced and IBA was incorporated and that were incubated exposing them to solar radiation during part of the rooting time had appropriate morphological characteristics that ensure greater survival during *ex vitro* acclimatization. In addition, the amount of inorganic salts in the culture medium was reduced and incubating with solar radiation is an alternative to reduce micropropagation costs.

Conclusions

All agave shoots that were established for *in vitro* rooting in culture media with 60% inorganic salts formed adventitious roots compared to shoots in culture media with 80 and 100% concentration of inorganic salts. Shoots established in culture medium with the IBA auxin formed more adventitious roots than shoots in culture medium without auxin. The best condition for rooting *A. nussaviorum* shoots was in culture medium with 60% MS inorganic salts and 0.5 or 1 mg L^{-1} of IBA.

The shoots incubated in the laboratory developed higher than the shoots incubated in the greenhouse, but the latter developed thicker and stiffer leaves, purple pigmentation on the underside, morphological characteristics that would facilitate their adaptation in an *ex vitro* environment. Therefore, the pre-acclimatization process in the greenhouse is recommended since it improves the quality of the plants *in vitro*.

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Shoots of *Agave nussaviorum* García-Mend. rooted by varying inorganic salts, IBA and incubation

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