Growth regulators, culture media and antibiotics in the in vitro shoot regeneration from mature tissue of citrus cultivars

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Abstract – The objective of this study was to evaluate the effects of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) combinations, basal media and beta-lactam antibiotics on in vitro organogenesis from mature stem segments of 'Pêra', 'Valência' and 'Bahia' sweet oranges and 'Cravo' rangpur lime. For induction of shoot regeneration, the segments of the four cultivars were placed on Murashige and Skoog (MS) medium containing the following BAP/NAA concentrations: 0.0/0.0; 0.25/0.0; 0.25/0.25; 0.5/0.0; 0.5/0.5; 1.0/0.0; 2.0/0.0; 2.0/0.25; 2.0/0.5; and 2.0/1.0 mg L^{-1}. In order to test the influence of the culture media on shoot-bud induction, (MS), Murashige and Tucker (MT), and woody plant medium (WPM) formulations were evaluated, associated with the best combination of plant growth regulators obtained in the previous experiment. The influence of four beta-lactam antibiotics (timentin, cefotaxime sodium salt, meropenem trihydrate and augmentin) on shoot regeneration was determined. Better regeneration responses were achieved when internodal segments were cultured onto MS-based medium with 500 mg L^{-1} cefotaxime with the following BAP/NAA concentrations: 0.5 + 0.25 mg L^{-1} for 'Cravo', 1.0 + 0.25 mg L^{-1} for 'Valência' and 'Bahia', and 1.0 + 0.5 mg L^{-1} for 'Pêra'. Genotype, growth regulators, basal media and beta-lactam antibiotics affect the morphogenetic response in mature tissues of citrus.

Index terms: *Citrus limonia*, *C. sinensis*, adult tissues, antibiotics, organogenesis.

Reguladores de crescimento, meio de cultura e antibióticos na regeneração in vitro de tecidos maduros de cultivares de citros

Resumo – O objetivo deste estudo foi avaliar o efeito de combinações de 6-benzilaminopurina (BAP) e ácido α-naftalenoacético (NAA), meios de cultura e antibióticos beta-lactâmicos sobre a regeneração in vitro de segmentos internodais de laranja doce ('Pêra', 'Valência' e 'Bahia') e limão 'Cravo'. Para a indução de brotações, os explantes internodais foram cultivados em meio Murashige e Skoog (MS) contendo as seguintes combinações de BAP/ANA: 0,0/0,0; 0,25/0,0; 0,25/0,25; 0,5/0,0; 0,5/0,5; 1,0/0,0; 2,0/0,0; 2,0/0,25; 2,0/0,5; e 1,0/2,0 mg L^{-1}. A fim de testar a influência dos meios de cultura na organogênese, as formulações (MS), Murashige e Tucker (MT) e "woody plant medium" (WPM) foram avaliadas, associadas à melhor combinação de reguladores de crescimento obtida no experimento anterior. Foram utilizados quatro antibióticos beta-lactâmicos (timentin, cefotaxima, trihidrato de meropenem e augmentin), em diferentes concentrações, para avaliar sua influência na regeneração in vitro. As melhores respostas foram obtidas quando os explantes foram cultivados em meio MS com 500 mg L^{-1} de cefotaxima com as seguintes combinações BAP/ANA: 0,5 + 0,25 mg L^{-1}, para 'Cravo'; 1,0 + 0,25 mg L^{-1}, para 'Valência' e 'Bahia'; e 1,0 + 0,5 mg L^{-1}, para 'Pêra'. Genótipos, meios de cultura e antibióticos beta-lactâmicos afetam a resposta morfogenética de tecidos maduros de citros.

Termos para Indexação: *Citrus limonia*, *C. sinensis*, antibióticos, organogênese, tecido adulto.

Introduction

Several methods for genetic transformation of citrus have been described in the literature, but so far the most effective are those using *Agrobacterium*-mediated transformation of juvenile materials, such as zygotic embryos, hypocotyls, epicotyls, and cotyledons (Fleming et al., 2000; Al-Bahrany, 2002; Costa et al., 2002; Oliveira et al., 2009). Plants regenerated from these explant sources have long juvenile stages before initial fruit production, and many years are necessary before evaluating the horticultural and commercial traits introduced into the transgenic plants. An ideal protocol for citrus plant transformation should be based on the use of mature tissues as explant sources, circumventing the juvenile phase and permitting the

analysis of the introduced traits in a relatively short period of time (Cervera et al., 2000; Peña et al., 2001; Almeida et al., 2003; Cervera et al., 2008; Rodríguez et al., 2008).

For successful genetic transformation of mature tissue, the first step is the establishment of an efficient plant regeneration system, since in vitro culture using mature tissue as explants is still far from routine (Almeida et al., 2003; Rodríguez et al., 2008). Reasons for this include the relatively low responsiveness of woody plants to exogenous growth regulators and the failure of standard surface sterilization techniques (Cervera et al., 2008). A culture medium with optimal mineral supply and combination of plant growth regulators will increase the success for recovery of transgenic citrus plants from transformed cells. Furthermore, the addition of antibiotics to the culture medium may help to eliminate contamination by bacteria, which hinder the in vitro establishment of explants from mature tissues.

Little is known about the effects of cultivars, growth regulators, basal media and beta-lactam antibiotics on in vitro shoot regeneration from mature citrus tissue. Beta-lactam antibiotics, such as carbenicillin and cefotaxime, are the most commonly used antibiotics in plant transformation protocols, since they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes (Bhau & Wakhlu, 2001; Yu et al., 2001). It has been speculated that some antibiotics may act as regulators of morphogenic development, and that they can enhance the in vitro response (Humara et al., 1999; Costa et al., 2000; Danilova et al., 2004).

The objective of this study was to evaluate the effects of different 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) combinations, basal media and beta-lactam antibiotics on in vitro shoot regeneration from mature stem segments of 'Pêra', 'Valência' and 'Bahia' sweet oranges and 'Cravo' rangpur lime.

Materials and Methods

The experiments were carried out in the plant tissue culture laboratory (Bioagro) at Universidade Federal de Viçosa, Minas Gerais, Brazil, from March to June 2005. Internodal segments, approximately 1.0–1.5-cm long, from greenhouse-grown adult plants of 'Pêra', 'Valência', and 'Bahia' sweet oranges [*Citrus sinensis* (L.) Osbeck] and 'Cravo' rangpur lime (*Citrus limonia* Osbeck) were used as sources of the mature explants. Rejuvenation was applied to facilitate in vitro culture of explants from mature tissue, by grafting buds into juvenile rootstocks. These mother plants were drastically pruned to stimulate the sprouting of the basal buds, which could retain juvenile characters. These plants were regularly sprayed with the fungicide Benlate (Benomyl) at 1% (v/v) to prevent contamination during in vitro culture. Vigorous newly elongated lateral branches, at least 12-inch long, were collected in a semi-hardened stage. Under aseptic conditions, elongated lateral branches were sterilized at the surface using 70% (v/v) ethanol for 2 min, followed by immersion in a 5% (v/v) commercial solution of sodium hypochlorite containing 0.1% (v/v) Tween-20 for 20 min, and sequentially rinsed in sterile distilled water.

The segments of the four cultivars were placed on MS (Murashige & Skoog, 1962) medium containing 14 combinations of BAP/NAA concentrations: 0.0/0.0, 0.25/0.0; 0.25/0.25, 0.5/0.0; 0.5/0.5; 1.0/0.0; 2.0/0.0; 2.0/0.25; 2.0/0.5 and 2.0/1.0 mg L^{-1}, for induction of shoot regeneration. Unless otherwise stated, all media were supplemented with 3% sucrose (w/v) and 0.65% (w/v) agar, with pH adjusted to 5.7±0.1 prior to autoclaving. The media were poured (25 mL aliquots) into sterile 90x15-mm Petri dishes. Explants were initially incubated in the dark at 26±2°C for 30 days and, then, transferred to a 16/8-hour (light/dark) photoperiod, and 36 µmol m^{-2} s^{-1} light radiation provided by two 20 W white fluorescent lamps for more 30 days.

In order to test the influence of the culture media on shoot-bud induction, three formulations were evaluated: MS, MT (Murashige & Tuker, 1969) and woody plant medium (WPM) (Lloyd & McCown, 1980). The best combination of plant growth regulators associated with the four genotypes was used in the media formulation: 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Cravo', 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Valência' and 'Bahia', and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA for 'Pêra'. Explants were initially incubated in the dark at 26±2°C for 30 days and, then, transferred to a 16/8-hour (light/dark) photoperiod, and 36 µmol m^{-2} s^{-1} light radiation provided by two 20 W white fluorescent lamps for more 30 days.

Elongated shoots obtained in all cultivars (1–1.5-cm long) were excised and subcultured on MS medium supplemented with 0.5 mg L^{-1} NAA for root induction during 60 days. Rooted shoots were transplanted to plastic cups containing sterile soil, sand and vermiculite (1:1:1, v/v/v) and were placed on illuminated shelves under 24 µmol m^{-2} s^{-1} irradiance, provided by two 20 W white fluorescent lamps. The plants remained under this condition for 45 days, and then they were transferred to greenhouse for additional 60 days.

The influence of beta-lactam antibiotics on shoot regeneration was determined through the best combinations of cultivar, culture media and growth regulators obtained in the previous experiments: 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Cravo', 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Valência' and 'Bahia' and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA for 'Pêra'. Four beta-lactam antibiotics were tested: timentin (GlaxoSmithKline, Rio de Janeiro, Brazil) (300 and 500 mg L^{-1}), cefotaxime sodium salt (Novafarma, Anápolis, Brazil) (250 and 500 mg L^{-1}), meropenem trihydrate (ABL, Cosmópolis, Brazil) (25, 50, 75 and 100 mg L^{-1}) and augmentin (GlaxoSmithKline, Budapest, Hungary) (250 and 500 mg L^{-1}). The antibiotics were added to the culture medium after autoclaving. Explants were incubated on 25-mL aliquots of semisolid medium under the same conditions, as previously described.

For all experiments described above, the cultures were sub-cultured after every 15–20 days on shoot induction media to obtain good growth. After culture for eight weeks, percentage of explants forming shoots and number of shoots per explant were recorded and statistically compared in each factor separately, following a completely randomized design. Twenty shoots were used for plate and five plates per experiment. Each experiment was repeated twice. Data were submitted to analysis of variance, and means were compared by t test or the Tukey-Kramer multiple comparison test at 1% probability.

Results and Discussion

Plant regeneration via direct and indirect organogenesis was obtained on the surface of the cut zone from cultured internodal explants of all four citrus cultivars tested. Small amounts of compact callus appeared on the cut surface within two weeks of culture, in segments incubated in darkness. Shoot buds differentiated from these calluses two weeks after transferring the cultures to the light.

Significant differences were observed for cultivars, basal media and antibiotics concentrations, in both percentage of explants forming shoots and average number of shoots per explants (Table 1). All null hypotheses on the effects of interaction between cultivars x BAP/NAA combinations, cultivars x antibiotics concentrations or cultivars x culture media were accepted. The absence of significance in the last interaction indicates that the cultivars did not differ in the various basal medium formulations.

Regeneration increased as the level of BAP in the culture medium increased from 0.25 to 1.0 mg L^{-1}, irrespective of the citrus cultivar and NAA concentration (Table 2). At 2.0 mg L^{-1} BAP, with or without NAA, the percentage of explants forming shoots decreased in most of the assessed cultivars. The combination of BAP and NAA was essential for maximum shoot production in all cultivars, although the balance between these

Table 1. Factorial analysis of variance for the percentage of explants forming shoots and average number of shoots per explant, in internodal segments of *Citrus sinensis* and *C. limonia* cultured for 60 days in different plant growth regulators concentrations (PGRC), basal culture media (BCM) or antibiotic concentrations (AC).

Source of variation	Explants forming shoots (%)		Nº of shoots per explant	
	DF	Mean square	DF	Mean square
Cultivars	3	1,745.8258**	3	0.5819**
PGRC	13	5,418.6199**	13	6.1034**
Cultivars x PGRC	39	466.3410**	39	0.9650**
Errors	224	134.5632	224	0.1669
CV (%)		13.224		39.224
Cultivars	3	739.9805**	3	1.0383**
BCM	2	1,263.2918**	2	4.3442**
Cultivars x BCM x PGRC	6	95.4814	6	0.1772
Errors	36	135.8773	36	0.1843
CV (%)		4.36		2.36
Cultivars	3	1,843.4090**	3	0.2024**
AC	9	5,884.0776**	9	11.3098**
Cultivars x AC	27	527.9170**	27	0.6608**
Errors	117	175.2750	117	0.2240
CV (%)		11.345		18.345

**Significant at 1% probability.

growth regulators for optimal shoot regeneration varied among cultivars. For 'Cravo', the highest number of shoots per explant was obtained in 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA, whereas 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA was the best combination for 'Valência' and 'Bahia'. 'Pêra' produced more shoots per explant in 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA.

Among cultivars, 'Cravo' showed a higher percentage (72.7%) of shoot regeneration and number of regenerated shoots per explant (3.1), followed by 'Pêra' (61.8%, and 2.5 shoots per explant) and 'Bahia' (65.4%, and 2.2 shoots per explant). 'Valência' (54.5%, and 2.1 shoots per explant) had the lowest shoot regeneration and no bud formation when BAP and NAA were absent.

Plant regeneration of explants from mature citrus tissue has been reported before only for 'Pineapple', 'Pêra', 'Valência', 'Hamlin' and 'Natal' (Cervera et al., 1998; Almeida et al., 2003; Kobayashi et al., 2003). Tissue regeneration had not been reported yet for 'Bahia', the most important edible orange in Brazil and in the USA (Washington navel orange), and 'Cravo' rangpur lime, an important citrus rootstock.

It has previously been reported that, differently from most juvenile explants, explants from mature citrus plants require balanced combinations of BAP and NAA in the culture media for maximum shoot regeneration (Almeida et al., 2003; Rodríguez et al., 2008). The present work corroborates these findings and further demonstrates that the optimal hormone concentrations are specific to cultivar, as it has been previously reported for juvenile explants (Bordón et al., 2000; Costa et al., 2004; Mendes et al., 2008).

Explants cultured in MS and MT media stimulated more morphogenesis than the WPM medium, irrespective of the cultivar analyzed (Table 3); however, WPM induced larger shoots than the MS and MT basal media. Similar observations have been reported for 'Pêra' (Kobayashi et al., 2003) and 'Clementine' mandarin (Cervera et al., 2008) using mature tissue as explant sources. The composition of the culture media used for citrus tissue culture is usually based on the nutrients and vitamins of MS and MT media, although WPM has been successfully used for tissue culture of recalcitrant woody species (Lloyd & McCown, 1980). It has been suggested that the lower

Table 2. Effect of 14 combinations of benzylaminopurine (BAP) and naphthalene acetic acid (NAA) concentrations on shoot organogenesis from internodal stem segments of citrus cultivars, after 60 days in MS-based culture medium[1].

BAP/NAA (mg L^{-1})	Number of shoots per explant				Explants forming shoots (%)			
	'Pêra'	'Valência'	'Bahia'	'Cravo'	'Pêra'	'Valência'	'Bahia'	'Cravo'
0.0/0.0	0.19 e	0.00e	0.09e	0.12e	9.10e	0.00c	5.45f	10.90e
0.25/0.0	1.00cd	0.40cde	0.94de	1.10bcde	29.10cd	16.36bc	18.18ef	41.81cd
0.25/ 0.25	0.60de	0.27de	0.76cde	0.74cde	16.40de	14.54bc	21.82def	30.90cde
0.50/0.0	1.18bcd	1.07abcde	1.10bcde	2.37ab	47.26abc	36.36ab	54.54abc	65.45ab
0.50/0.25	1.27bcd	0.98abcde	1.23abcd	3.05a	56.36ab	30.90ab	45.45bcd	72.72a
0.50/0.50	0.94cd	0.88abcde	0.90bcde	1.77bc	43.63bc	32.72ab	41.81bcde	58.17abc
1.0/ 0.0	1.39bcd	1.83ab	1.74abc	2.03abc	69.10a	56.36a	70.90a	63.63ab
1.0/0.25	1.94ab	2.12a	2.23a	1.99bc	67.27a	54.54a	65.44ab	60.00ab
1.0/0.50	2.46a	1.99a	1.94ab	1.47bcd	61.81ab	52.72a	63.63abc	58.17abc
1.0/1.0	1.05cd	0.60bcde	0.88bcde	0.67cde	52.72ab	51.51a	40.00cde	29.08de
2.0/0.0	1.25bcd	1.28abcd	1.08bcde	1.01bcd	56.36ab	56.36a	60.00abc	38.18cde
2.0/0.25	1.63abc	1.45abc	1.38abcd	0.70cde	58.17ab	50.36a	56.36abc	32.72cde
2.0/0.50	1.56bc	1.36abcd	1.12abcd	0.80cde	56.60ab	45.45a	52.72abc	29.08de
2.0/1.0	2.05ab	1.43abc	1.17abcd	0.54de	58.18ab	43.63a	50.90abc	25.45de

[1]Means followed by equal letters within a cultivar do not differ by the Tukey's test, at 5% probability.

Table 3. Basal medium – Murashige and Skoog (MS), Murashige and Tucker (MT), and woody plant medium (WPM) – influence on shoot organogenesis from mature internodal segments of four citrus cultivars, after 60 days of in vitro culture[1].

Basal medium	Number of shoots per explant				Explants forming shoots (%)			
	'Pêra'	'Valência'	'Bahia'	'Cravo'	'Pêra'	'Valência'	'Bahia'	'Cravo'
MS	2.63a	1.98a	2.06a	3.41a	63.63a	49.09a	63.63a	75.54a
MT	2.27a	1.61a	2.03a	3.40a	65.45a	45.45a	58.18a	69.08a
WPM	1.29b	1.01b	1.18b	1.45b	41.81b	38.18b	45.45b	47.27b

[1]Means followed by equal letters within a cultivar do not differ by the Tukey's test, at 5% probability.

concentration of nitrogen and potassium in WPM could account for the differences in shoot size (Kobayashi et al., 2003).

Bacterial contamination during in vitro culture of mature internodal segments from greenhouse-grown mother plants is usually responsible for the loss of explants cultured on antibiotic-free medium. However, addition of antibiotics to the culture medium requires a careful evaluation of their effects on plant regeneration, since several reports have shown that they could have positive or negative effects on in vitro morphogenesis (Costa et al., 2000; Tang et al., 2004; Mendes et al., 2009).

Buds from mature explants, irrespective of the cultivar, were significantly influenced by the type and concentration of the antibiotics. Among the evaluated beta-lactams, the best responses of the mature explants were obtained with 500 mg L^{-1} cefotaxime, since it either promoted ('Pêra', 'Valência' and 'Bahia') or had no effect on shoot regeneration ('Cravo') (Table 2). Buds and shoots did not show any kind of burning. In 'Pêra', the mean number of shoots per explant increased from 2.5 (Table 2) to 4.03 (Table 4). In 'Valência' and 'Bahia', the same concentration of cefotaxime also increased shoot formation from 54.5 and 65.44% (Table 2) to 83.62 and 76.24% (Table 4), respectively. All other antibiotics had negative effects on shoot regeneration, except for timentin at 500 mg L^{-1} and augmentin at 500 mg L^{-1}, which did not inhibit shoot regeneration in 'Pêra', 'Valência' or 'Bahia'. The lowest concentrations of beta-lactam antibiotics (25 mg L^{-1} meropenem, 300 mg L^{-1} timentin and 250 mg L^{-1} augmentin) were not able to suppress bacterial growth. In explants cultured on 250 mg L^{-1} augmentin, bacteria growth was observed within seven days of culture.

These observations were quite surprising, since it has been recently reported that, in contrast to meropenem and timentin, cefotaxime caused a negative effect on shoot regeneration from epicotyl explants of citrus (Mendes et al., 2009). It has also been recently reported that meropenem was more effective against *Agrobacterium* and also improved transformation efficiencies in tobacco, tomato and rice, in comparison to cefotaxime and carbenicillin (Ogawa & Mii, 2004, 2005, 2007).

Stimulatory effects of cefotaxime on plant morphogenesis have been also reported for several species, such as *Pinus pinea* (Humara & Ordás 1999), *Coryphantha elephantidens* (Bhau & Wakhlu, 2001) and *Zea mays* (Danilova & Dolgikh, 2004). Some possibilities used to explain the stimulatory effect of antibiotics on plant morphogenesis include their chemical structure, which may mimic plant growth regulators (Nakano & Mii, 1993), or their degradation by-products, which may generate metabolites with plant growth regulator activity (Mathias & Mukasa, 1987).

Table 4. Influence of beta-lactam antibiotics on shoot organogenesis in internodal segments of *Citrus sinensis* and *C. limonia* cultivars cultured for 60 days in MS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Cravo', 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Valência' and 'Bahia' and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA for 'Pêra'[(1)].

Doses (mg L^{-1})	Nº of shoots per explant				Explants forming shoots (%)			
	'Pêra'	'Valência'	'Bahia'	'Cravo'	'Pêra'	'Valência'	'Bahia'	'Cravo'
				Meropenem				
25	0.84de	0.55de	0.79de	1.09cde	34.54abcd	14.54ef	47.04abcd	39.99d
50	1.40bcd	1.60abcd	1.55bc	1.77bcd	52.72abc	54.54bcd	58.52abc	63.63abcd
75	1.56bcd	1.54abcd	1.49bc	1.66bcd	49.08abcd	65.44ab	52.14abcd	65.44abc
100	0.60de	1.19bcd	0.98de	0.74de	21.81cde	36.36cde	31.72cde	49.08bcd
				Timentin				
300	0.38de	1.26abcd	1.15bcd	1.14cd	25.45bcde	54.54bcd	35.54bcde	43.63cd
500	2.20bc	2.09ab	1.98bc	2.36ab	56.35ab	59.99abc	64.25ab	69.08ab
				Cefotaxime				
250	0.75de	1.42abcd	1.08bcd	1.05cde	19.99de	54.54bcd	29.79de	43.63cd
500	4.03a	2.50a	3.79a	3.03a	58.17a	83.62a	76.24a	76.35a
				Augmentin				
250	1.07cde	0.68de	0.84de	0.88cde	54.54ab	27.27def	63.57ab	43.63cd
500	2.41b	1.63abcd	2.11b	2.07abc	52.72abc	56.35abc	24.77abc	56.35abcd

[(1)]Means followed by equal letters within a column do not differ by Tukey's test, at 5% probability.

Conclusions

1. Genotype, growth regulators, basal media and beta-lactam antibiotics affect the morphogenetic response in mature tissues of citrus.

2. The protocol using MS medium supplemented with 500 mg L^{-1} cefotaxime and 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Cravo', 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} NAA for 'Valência' and 'Bahia' and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA for 'Pêra' is efficient in plant regeneration from mature tissue of these citrus cultivars.

3. None of beta-lactams antibiotics tested is able to completely suppress bacterial contamination; however, the use of 500 mg L^{-1} cefotaxime in the culture medium is advantageous both to control bacterial contamination and to influence positively the morphogenetic process, especially in sweet orange.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico and to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, for financial support.

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Received on December 17, 2009 and accepted on April 26, 2010